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UNIVERSITY OF ALBERTA

MODIFYING IMMUNE RESPONSES THROUGH ANTIGEN DELIVERY APPROACHES

BY

M. E. CHRISTINE LUTSIAK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
AND RESEARCH IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

Faculty of Pharmacy and Pharmaceutical Sciences
Edmonton, Alberta, Canada
[illegible]

"Be patient toward all that is unsolved in your heart, and try and love the questions themselves, like locked doors, or like books that are written in a very foreign tongue."

- R. M. Rilke

"A scientist must be willing to live a life that is 90% depression and 10% elation- on a good day."

- Polly Matzinger

UNIVERSITY OF ALBERTA

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Faculty of Pharmacy and Pharmaceutical Sciences
Edmonton, Alberta, Canada
Fall, 2002

University of Alberta
Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Modifying Immune Responses Through Antigen Delivery Approaches** submitted by **M. E. Christine Lutsiak** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences.

Abstract

Antigen delivery systems influence the nature of immune responses. This dissertation summarizes research performed on two systems, liposomes and poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres, in the context of therapeutic vaccines designed to activate cell-mediated immune responses (CMI) against cancer and chronic viral infections.

A series of liposomal and nanosphere formulations of therapeutically relevant peptides were characterized with respect to their size, peptide content, and retention of the peptide. Methods for quantification of peptides in these formulations were developed and optimized. The ability of the delivery systems to bias immune responses towards CMI was evaluated using $HBcAg_{129-140}$ as a model antigen. This peptide, when encapsulated in liposomes or nanospheres along with monophosphoryl lipid A (MPLA), an immunomodulator, induced a T helper 1 (Th1)-type response in mice, despite its tendency to induce a Th2 response when delivered in other formulations. The nanosphere formulation successfully induced a Th1-type response even after the immune system was primed for a Th2-type response.

In order to establish an effective response, therapeutic vaccines must deliver antigen to dendritic cells (DC). The ability of human DC to phagocytose PLGA nanospheres was investigated *in vitro* and the uptake characterized. DC were highly capable of phagocytosing nanospheres, although this ability was affected by the day in culture. At day 3, but not day 8, phagocytosis

resulted in upregulation of major histocompatibility complex class II and CD86 molecules. No consistent effects of MPLA on DC uptake of particles were observed.

The ability of DC to stimulate T cell responses to a MUC1 mucin peptide, a candidate for the immunotherapy of $MUC1^+$ tumors, after phagocytosing nanospheres containing the peptide and MPLA was examined *in vitro*. DC stimulated a strong T cell proliferative response only when peptide and MPLA were co-administered in the same particle. These results demonstrate that nanospheres are suitable for efficient loading of DC with antigens so as to break T cell tolerance to a self-antigen.

This research demonstrates that liposomes and PLGA nanospheres are efficient antigen delivery systems for induction of CMI and are important candidates for the formulation and delivery of therapeutic vaccines for cancer and chronic viral infections.

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List Of Abbreviations

ABTS	2, 2'-azino-di(3-ethyl-benzthiazoline sulfonate)
APC	antigen presenting cells
BSA	bovine serum albumin
C	Celsius
CD40L	CD40 ligand
CFA	complete Freund's adjuvant
CHOL	cholesterol
CMI	cell mediated immunity
Con A	concanavalin A
CpG	cytosine-phosphate-guanosine
CPM	counts per minute
CTL	cytotoxic T lymphocyte
Cyto B	cytochalasin B
DC	dendritic cells
DMPG	dimyristoyl phosphatidyl glycerol
DOPE	dioleoyl phosphatidyl ethanolamine
DPM	decays per minute
DPPC	dipalmitoyl phosphatidyl choline
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor
HBcAg	hepatitis B core antigen
$HBcAg_{110-140}$	hepatitis B core antigen residues 110-140
$HBcAg_{120-140}$	hepatitis B core antigen residues 120-140
$HBcAg_{126-140}$	hepatitis B core antigen residues 126-140
$HBcAg_{129-140}$	hepatitis B core antigen residues 129-140
HBsAg	hepatitis B surface antigen
HBSS	Hank's balanced salt solution

HBV	hepatitis B virus
HI	humoral immunity
HPLC	high performance liquid chromatography
HLA	human leukocyte antigen
HS	human serum
IFA	incomplete Freund's adjuvant
IFN-γ	interferon-gamma
IL	interleukin
ISCOMS	immunostimulatory complexes
i.p.	intraperitoneal
LMP	low molecular mass polypeptide
LT	lymphotoxin
Mabs	monoclonal antibodies
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MPLA	monophosphoryl lipid A
MPS	mononuclear phagocytic system
MW	molecular weight
Mϕ	macrophages
NK	natural killer
NS	nanospheres
OD	optical density
PBLs	peripheral blood leukocytes
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PKC	phosphokinase C
PLGA	poly(D,L-lactic-co-glycolic acid)
PVA	polyvinyl alcohol
RER	rough endoplasmic reticulum
RPMI-10HS	RPMI media supplemented with 10% human serum
s.c.	subcutaneous
SI	stimulation index

TAA	tumor-associated antigen
TAP	transporter of antigenic peptides
TCR	T cell receptor
Th	T helper
THF	tetrahydrofuran
TMB	3, 3', 5, 5', tetramethylbenzidine
TMR	tetramethylrhodamine
TPBS	0.05% (v/v) Tween 20/PBS
UV	ultraviolet
W/o/w	water-in-oil-in-water

Amino Acids

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Chapter One

Introduction

1.1 Introduction

One of the most successful and widely used types of medical intervention is immunoprophylaxis. In addition to their traditional use as preventive agents, vaccines may find new applications as therapeutic agents. Preventative vaccines have virtually eliminated several infectious diseases, including polio and small pox. In contrast, immunotherapy of established malignancies or chronic infections is not yet in widespread clinical use. The relative lack of efficacy of such therapeutic vaccines may be attributed to the immune evasion mechanisms developed by viruses and cancer cells. A more thorough understanding of the functioning of the immune system allows for improved design of existing vaccines and the development of new vaccines against diseases such as HIV or cancer. The continued refining of vaccine formulations depends on the development of novel antigen delivery systems and adjuvants that improve the potency and safety profiles of new and existing vaccines. Biodegradable and biocompatible liposomes and polymeric nanospheres have been evaluated for the encapsulation and delivery of a variety of antigens (1-5). This dissertation evaluates liposomes and poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres as delivery systems for peptide antigens in the treatment of viral infections and malignancies. The following chapter provides the rationale for this research and discusses the mechanisms involved in eliciting an effective immune response against cancer and viral antigens.

1.2 Characterization of Immune Responses

1.2.1 Cellular Versus Humoral Immune Responses

In order to be effective, vaccines must activate the immune system to establish one or both of the two types of acquired immune response: humoral immunity (HI) or cell mediated immunity (CMI). In general, humoral immune responses are effective against extracellular microorganisms (6-8), while cell mediated immune response are required for the eradication of intracellular pathogens (9, 10) and cancer (11).

The humoral response is characterized by the production of antibody molecules by B lymphocytes. These cells become activated after foreign antigen, in its native conformation, binds with the antibody molecules on their cell surface. After activation, B cells secrete soluble antibody with the same antigen-specificity as the parent cell. Soluble antibody aids in the elimination of pathogens through several mechanisms: binding to virus and preventing cell entry; activation of the complement system resulting in lysis of the organism; acting as opsonins to enhance the phagocytosis of the microorganisms; and binding to Fc receptors on natural killer (NK) cells or macrophages (Mϕ) to instigate antibody-dependent cell-mediated cytotoxicity (ADCC).

Cell-mediated immunity consists of activated antigen-specific cytotoxic T lymphocytes (CTL) that can kill infected or malignant cells. Activation of a cell-mediated response also involves antigen-specific regulatory cells, T helper (Th) cells and non-antigen-specific cells including Mϕ, NK cells, and neutrophils (12). CTL are CD8$^+$ cells that recognize antigen fragments or epitopes in association with Class I major histocompatibility complexes (MHC) on the surface of altered self-cells. CTL bind to the peptide-MHC class I complex and, in the presence of a costimulatory signal and assistance from Th cells, become activated to effector cells that can kill target cells. CMI is effective against intracellular pathogens, such as viruses, or cancerous cells but is less effective against extracellular microorganisms.

1.2.2 T helper 1 Versus T helper 2

Th cells play a regulatory role in the development, maintenance, and deactivation of the immune response. Th cells are required for the initiation of both humoral and cell mediated responses. The type of response is dependent on the type of Th cells activated by the antigen (13-15). Th cells are CD4$^+$ cells that recognize epitopes in conjunction with MHC class II on the surface of antigen presenting cells (APC). Activation occurs following

recognition of the peptide-MHC complex and costimulation. Upon activation, Th cells become effector cells that secrete cytokines, proteins that regulate the immune response.

The two subsets of Th cells have diverse functions and can be identified by their differing cytokine profiles (16). The Th1 subset assists in the development of cellular responses and secretes interleukin (IL)-2, lymphotoxin (LT) and interferon-gamma (IFN-γ) (15, 17-20) while the Th2 subset aids humoral responses and secretes IL-4, -5, -6, and -10 (18-20). Each Th subset downregulates the other and self-stimulates in an autocrine manner; IFN-γ stimulates Th1 cells and inhibits Th2 cells (15) while IL-10 stimulates Th2 cells and inhibits Th1 cells (21). Because of this ability to selectively stimulate the same kind of response and inhibit the opposing type of response, a Th response, and therefore the overall immune response, has the potential to become a self-sustaining reaction of one type. Typically, the response to a pathogen is a mixture of cellular and humoral responses, the balance of the two located on a continuum between the opposing responses. In certain disease states or after certain modes of immunization, the response can be polarized to one side or the other.

1.2.2.1 Factors Contributing to Th Cell Differentiation

Many factors affect which type of Th cell response is generated against the antigen. The aggregate of all factors determines the type of immune response elicited. These factors can be related to the antigen, such as source or dose; to the host, such as age or genetic background; or to events at the cellular level, such as cytokine environment or costimulatory molecules. The mechanism(s) by which these factors can alter the type of response to an antigen is under investigation.

One of the important factors affecting the development of the immune response is the source of the antigen. For example, microbial products that accompany antigens can significantly influence the immune response. Lipopolysaccharide, CpG oligonucleotides, and double-stranded RNA are all

molecules of microbial origin that influence the selection of the type of immune response. One hypothesis is that they may exert their role by causing the production of certain cytokines *in vivo*. The cytokine environment at the site of antigen exposure to the immune system can be crucial in the development of a response; IL-12 leads to the development of a Th1-type response while IL-4 leads to a Th2-type response. The cytokines may act by inducing the T cells to develop into a certain type of cell or they may simply be growth factors for T cells that have been preprogrammed to develop into a certain type. There is evidence, however, that rules out the T cells themselves as the deciding factor in which type of immune response is elicited in response to an antigen. T cells with the same T cell receptor (TCR) can be driven to different subtypes depending on the cytokine environment so both Th1 and Th2 cells share a common precursor (22).

Another factor that has been postulated as a major force in the development of a certain type of response is the type of costimulatory molecule involved in T cell activation, however, the evidence in this area is inconsistent. Several studies have demonstrated that CD80 (B7-1) engagement leads to a Th1-type response while CD86 (B7-2) binding leads to a Th2-type response; however, there have also been reports of CD86 molecules inducing both Th1- and Th2-type responses (23). The receptors for CD80 and CD86, CD28 and CTLA-4, have also been implicated in the Th1/Th2 choice; evidence suggests that signaling through CD28 leads to a Th2-type response (24, 25) while engagement of CTLA-4 inhibits the development of a Th2-type response (26) but these data are contradicted by other studies (22). In addition, it has been shown that different types of cell signaling through the TCR lead to different types of T helper responses (27). In particular, the stimulation of phosphokinase C (PKC) activation or the partial blockage of calcineurin in developing effector cells leads to a Th2-type response while the stimulation of a Ca^{2+} flux or the partial inhibition of PKC leads to a Th1-type response (28). It seems likely that altered signaling into the T cells through the TCR or the costimulatory molecules could act to affect the type of immune

response by regulating the secretion of IL-4, the expression of cell surface molecules, or the sensitivity of the cell to certain cytokines. The effect of the signals conducted through cell surface markers on the type of T helper response developed is an area for further investigation.

In the event that cytokine production and/or signaling into the T cell through costimulatory molecules or the TCR is the deciding factor in the type of response developed against an antigen, it would appear that the APC may play the deciding role in the development of T helper responses. It has been postulated that Th1-type responses are stimulated by DC1 cells and Th2-type responses by DC2 cells (29-31). In this instance, DC subsets would be defined by precursor cells, localization, or maturation state and external factors would be unable to alter the effect of the DC on the type of immune response. In contrast, it is likely that a single DC can prime for either a Th1-type or a Th2-type response depending on the nature of its activation. In this case, the critical role in Th1/Th2 development would be played by the antigen and the local environment of antigen exposure to the immune system. A recent study demonstrated that a single population of DC could elicit a Th1-type response to the influenza virus while eliciting a Th2-type response to the fetal calf serum in the culture medium (32). This evidence supports the view that DC can lead to the establishment of a Th1- or a Th2-type response, depending on other factors.

1.2.3 Therapeutic Relevance of the Type of Immune Response

Th1-type responses are protective against altered self-cells: cancerous cells or cells containing an intracellular pathogen (33-35). Extracellular organisms are eradicated by Th2-type responses. As has been shown in Leishmania (36, 37) and human immunodeficiency virus (HIV) infections (38), an inappropriate response is detrimental while an appropriate response is curative. Because of the self-sustaining nature of the Th1- and Th2-type responses, external assistance may be required to switch an established immune response to a more beneficial one. In an *in vitro* system using

human T cells, it has been shown that polarized Th2 cells can become Th0 or Th1 cells under certain circumstances, opening up investigations into therapeutically useful ways to alter an ongoing immune response *in vivo* (39).

1.3 Antigen Processing and Presentation

Unlike B cells, T lymphocytes do not recognize foreign molecules in their native conformation, instead, they recognize fragments of foreign antigen in association with self MHC molecules on the surface of self-cells. In order for an antigen to be presented to T cells, it must be processed and presented on either class I or class II MHC molecules. There are two pathways for antigen processing and presentation (40): the MHC class I pathway and the MHC class II pathway. The class I MHC pathway is usually the processing pathway for endogenous antigen, leading to CTL activation. The class I pathway is active in all nucleated cells of the body; consequently, all cells can present antigen to the CTL and be killed when necessary. In contrast, the class II MHC pathway is usually the processing pathway for exogenous antigen, leading to the activation of T helper cells. The class II pathway is active only in APC, which activate the regulatory T cells.

1.3.1 Antigen Presenting Cells

APC sample antigen in the periphery and carry it back to the lymph node where it is presented to T cells. After internalization of antigen, APC process and present it to CD4$^+$ T lymphocytes in association with class II MHC molecules. Effective processing and presentation of foreign antigens by APC is necessary for the activation of CMI. Vaccines must be formulated with this necessity for delivery to APC in mind, in addition, the type of APC internalizing and presenting the antigen is an important factor to consider. B cells, DC, and Mϕ all have the capability of processing and presenting antigen to T cells, however, their abilities vary. Because B cells are inefficient at pinocytosis and phagocytosis, they can only present antigen that has been internalized via their immunoglobulin receptors (41, 42). Conversely, the

Mϕ are highly phagocytic cells capable of processing and presenting a wide range of antigens in soluble or particulate form (42-44). Recent studies, however, have identified DC as the key antigen presenting cell (45).

Because of their constitutive expression of costimulatory molecules and MHC class I and II, and their rapid upregulation of high levels of these molecules upon activation, DC are the only APC capable of stimulating naïve T cells (46-49). In addition, the production of IL-12 by mature DC is a major factor in the induction of Th1-type responses (50, 51). Antigen acquisition by DC is important in determining the outcome in viral infections and cancer, where Th1-type responses are thought to be protective. Therefore, therapeutic vaccines for cancer and viral infections should be designed to deliver antigens to DC. DC loaded with cancer antigens *ex vivo* are now under investigation as therapeutic cancer vaccines and show encouraging results in cancer patients (52, 53). More efficient delivery of antigens to DC *ex vivo* and delivery of antigens to DC *in vivo* are both important goals for research into therapeutic vaccines for cancer and infectious disease.

1.3.2 Dendritic Cells

1.3.2.1 *In vitro* Culture of Dendritic Cells

Fully differentiated DC can be isolated from peripheral blood (54); however, they are found at a very low concentration, as only approximately 0.5% of peripheral blood mononuclear cells are dendritic cells (55). In contrast, it has been reported that adherent peripheral blood mononuclear cells, cultured in media containing granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4), will develop into DC with a yield of about 8% (55). These cultured cells are indistinguishable from freshly isolated DC of the myeloid lineage with respect to morphology, expression of cell surface markers, and T cell stimulatory function (55). Monocytes from the peripheral blood are induced to develop into DC and Mϕ by the presence of GM-CSF. IL-4 acts to completely suppress the expression of CD14 mRNA, preventing the development of Mϕ under these culture conditions (56).

1.3.2.2 Antigen Uptake by Dendritic Cells

DC capture and internalize antigen through several uptake pathways. First, DC are highly efficient at pinocytosis. The rate of fluid uptake by a single DC has been estimated at 1000-1500 μm^3 per hour or an entire cell volume every hour (57). This massive uptake allows the DC to concentrate antigen and generates efficient presentation of soluble antigens, even at concentrations in the picomolar range (45). Second, DC can uptake antigen through receptor-mediated endocytosis. They express the macrophage mannose receptor and DEC-205 (57), in addition to two types of Fcγ receptor (58). The third uptake mechanism used by DC is phagocytosis. When DC were first characterized as APC, they were thought to be non-phagocytic (59). More recently, they have been shown to phagocytose a variety of materials including microorganisms and latex beads (60, 61).

The ability of DC to internalize and process antigen is highly dependent upon the stage of DC differentiation (60, 62). Immature cells residing in the tissue are highly phagocytic and are less efficient at stimulating T lymphocytes. Following maturation, DC cease phagocytosis and evolve into efficient APC that express high levels of costimulatory molecules (60, 62).

1.3.2.3 Dendritic Cell Subsets

The high degree of heterogeneity among DC subsets may reflect the multiple functions of this cell type (63, 64). Studies in mice have suggested that DC subsets may have different roles in eliciting T cell responses to antigen. In these studies, $CD8^+$ DC were identified as promoting Th1-type responses while $CD8^-$ DC promoted Th2-type responses (30, 62). However, other data suggests that the function of DC is not preprogrammed but is modulated by environmental factors (66, 67). Experiments involving immunization with heat killed *Brucella abortus*, a Th1-inducing antigen, in mice, demonstrated that both $CD8^+$ and $CD8^-$ DC produced IL-12 in response to antigenic challenge (68), supporting the view that DC subsets are functionally plastic.

It is possible, however, that environmental factors during infection or post-vaccination can lead to the development of separate DC with separate functions.

1.3.3 Class I Presentation

Presentation on Class I MHC allows for the killing of any self-cell that has been altered through viral infection or malignancy. Class I MHC, located on all nucleated cells, presents peptides of 8-9 amino acids in length to CD8$^+$ T cells. In the human population, there are many alleles for Class I MHC, leading to proteins with differing binding cleft characteristics. This genetic diversity affects which peptides an individual can present and respond to and must be taken into account when designing therapeutic vaccines.

1.3.3.1 Endogenous Antigen

Endogenous antigen is degraded into peptides in the cytoplasm of a cell by a proteolytic complex, the low molecular mass polypeptide (LMP) (69, 70) (Figure 1-1). Peptides are transported into the rough endoplasmic reticulum (RER) by TAP, transporter of antigenic proteins. Once in the RER, peptides with 8-9 residues bind to the MHC class I molecule through the anchor motifs, specific anchor residues that are preferentially bound by the different class I MHC allelic products. Finally, the peptide-MHC complex is transported to the cell surface.

1.3.3.2 Exogenous Antigen

In order for exogenous antigen to enter the class I MHC antigen processing pathway, the antigen must leave the endosomal/lysosomal compartment and enter the cytoplasm. It has been shown that all three of the professional antigen presenting cells transfer exogenous antigen across the endosomal/lysosomal membrane to the cytosol where it is processed through the same pathway as the endogenous antigen (71-74). Cytoplasmic delivery of an exogenous antigen is influenced by the nature of the antigen; in DC

and Mϕ, presentation of an exogenous antigen on class I MHC was enhanced when the antigen was in particulate form and internalized by phagocytosis (73, 75, 76). A TAP-independent pathway for the presentation of exogenous antigens on class I MHC has also been discovered. Processing along this pathway involves the hydrolysis of antigen in the endosome, followed by the direct loading of peptide onto class I MHC molecules (77).

1.3.4 Class II Presentation

Class II MHC molecules, located on APC, present peptides of 13-17 residues in length to CD4$^+$ T cells (78, 79). Presentation of antigen on MHC class II leads to the activation of Th cells, which are required for both humoral and cell mediated responses. As with MHC class I, there are numerous alleles in the population, affecting which peptides an individual can present to T lymphocytes.

After antigen is internalized by phagocytosis or endocytosis, it enters the class II processing pathway (Figure 1-1). The antigen progresses through increasingly acidic compartments, from the endosome to the endolysosome to the lysosome, where it is degraded into peptides. In the RER, newly synthesized MHC class II molecules associate with invariant chains, proteins that bind to the antigen-binding cleft of MHC class II and prevent the binding of peptides derived from intracellular antigens to the MHC class II molecules (80). The class II MHC-invariant chain complexes pass through the Golgi complex to the endocytic pathway. In the endocytic pathway, the invariant chains are degraded, exposing the antigen-binding cleft of the class II MHC molecules. Peptides derived from the phagocytosed antigen that contain appropriate anchor residues then associate with MHC class II molecules and the antigen-MHC complex is transported to the cell surface for presentation to T cells.

1.4 Modulation of Immune Responses

1.4.1 Adjuvants

An adjuvant is a substance (or combination of substances) that enhances or modulates the humoral or cellular immune response against an antigen (81). Adjuvants exert their effects through several possible mechanisms (81). Certain adjuvants perform by combining with the antigen to act as a depot, prolonging the time the antigen is available to the immune system. Other adjuvants generate the production of cytokines or enhance the levels of costimulatory molecules on APC (82-85). Finally, adjuvants can directly target immune cells by delivering antigen of a particulate nature to APC.

Conventional vaccines elicit a strong antibody response to an inactivated pathogen, usually in combination with the adjuvant alum. Changes in the nature of vaccine components and the development of therapeutic vaccines require the development of new adjuvants. There is significant interest in designing vaccines that can elicit a cell-mediated immune response against viral or cancer antigens, and the development of new adjuvants is an potentially useful strategy for achieving this objective.

It has proven difficult to develop new adjuvants for widespread clinical use. Safety is a major factor limiting the approval of new vaccine adjuvants. In addition, not all adjuvants perform in the same manner with all vaccines. Thus, the selection of an appropriate adjuvant relies on both the antigen and the type of response desired. These issues must be considered during the design of therapeutic vaccine formulations.

One approach to the rational design of adjuvants, particularly Th1-inducing adjuvants, is to identify microbial products that activate the immune system and modify the molecules for use in vaccine formulations. DC are activated by 'danger' signals provided by bacteria and viruses. These substances,

including unmethylated CpG oligonucleotides, double-stranded RNA, and lipopolysaccharides (LPS), are suitable starting points for the development of adjuvants (81). Unmethylated CpG oligonucleotides are commonly found in bacterial DNA and have been shown to be highly effective at inducing Th1-type responses (86, 87). CpG oligonucleotides have been shown to activate T, B, and NK cells, as well as Mϕ to produce proinflammatory cytokines (88, 89). CpG oligonucleotides have been demonstrated to be effective adjuvants for vaccination against infectious agents and cancer antigens (90-92). A hepatitis B vaccine using CpG oligonucleotides as the adjuvant is in clinical trials (86), indicating that the investigation of microbial products for use as adjuvants is a valuable area of investigation.

1.4.1.1 Alum

Aluminum salts, which work by forming a depot of the antigen at the site of injection (93), remain the standard adjuvant for use in humans (94). From the 1920's, when they were first used to enhance the immunogenicity of vaccines, to 1997, when MF59 was licensed for use with the influenza vaccine, alum was the only vaccine approved for human use (94). Alum has proven ineffective as an adjuvant for influenza vaccines and for various recombinant antigens and synthetic peptides (95). In addition, alum is a Th2-inducing adjuvant and is not suitable for the development of cell mediated responses (96).

1.4.1.2 Complete Freund's Adjuvant (CFA)

CFA is a Th1-inducing adjuvant (97). It consists of mineral oil along with killed mycoplasmic bacteria and is emulsified with antigen prior to injection. Its mechanism of action involves the formation of a depot of antigen as well as the non-specific activation of the immune system due to the microbial products included in the formulation. Due to safety concerns, CFA is not approved for use in humans and CCAC (Canadian Council on Animal Care) regulations prohibit its use in animals where it can be avoided. Incomplete Freund's adjuvant (IFA) does not contain the killed mycoplasmic bacteria and

functions as a somewhat safer adjuvant than CFA, but is not approved for general use in humans.

1.4.1.3 Monophosphoryl Lipid A (MPLA)

Because the immune system has evolved to respond to microbial products, these products or their derivatives are a logical starting point for the development of adjuvants. It is known that lipopolysaccharide (LPS) drives the maturation of DC (98). LPS, a constituent of the outer membrane of Gram$^-$ bacteria, is composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A (99). Because of its high toxicity, LPS is not itself a viable adjuvant candidate; however, the adjuvant activity of the molecule resides in the lipid portion of LPS and lipid A has come under investigation as a potential adjuvant. A derivative of lipid A, MPLA (Figure 1-2), lacking one of the phosphate groups, has a dramatically decreased toxicity over the parent molecule (100).

In a recent study, the effect of MPLA on human DC was examined with respect to cell surface marker expression and cytokine production. The upregulation of HLA-DR, CD80, CD86, CD40, and CD83 in response to MPLA was heterogeneous but, in all donors, at least three of the five markers were upregulated (101). In addition, IL-12 release by human DC was significantly enhanced by MPLA, however, the levels were less than those seen with LPS treatment (101). These data are congruent with MPLA-induced activation of DC.

In view of the fact that MPLA could lead to activation of DC, the ability of MPLA-treated DC to stimulate a T cell response was tested. MPLA enhanced the activation of allogeneic T cells by DC as evidenced by the production of IFN-γ and IL-5 in the culture supernatant (101). In addition, low doses of MPLA that proved insufficient to activate DC also induced strong T cell responses (101). This indicates a direct action of MPLA on the T lymphocytes. To further define the effect of MPLA on T cells, the expression

of CD40 ligand (CD40L) on the surface of T cells was investigated. The results demonstrated that MPLA enhanced the expression of CD40L on the surface of T cells (101). DC receive maturation and survival signals from T cells via the CD40/CD40L interaction. It is therefore apparent that MPLA acts on both DC and T cells, causing them to further activate each other.

MPLA incorporated in liposomes has been shown to be safe and effective for the induction of humoral (102) and cell mediated immunity (103). Furthermore, clinical trials have demonstrated the suitability of liposomal MPLA as an adjuvant for use in humans (104).

1.5 Current Vaccine Strategies

1.5.1 Inactivated Microorganisms

Traditional vaccines consist of inactivated or attenuated whole microorganisms or toxins often combined with alum. These antigens are highly immunogenic and, therefore, they do not require significant assistance to elicit an effective immune response. Because these vaccines contain whole microorganisms, it is possible for them to revert to an infectious form and cause, rather than prevent, disease. In addition, even without reversion, the weakened bacteria may still be strong enough to cause disease in immunocompromised patients such as the very young, the elderly, transplant recipients, and the chronically ill. As the number of patients in whom the use of traditional vaccines is contraindicated rises, the need for vaccine formulations containing alternative forms of antigens escalates.

1.5.2 Protein Antigen

Recombinant protein antigens are a type of subunit vaccine that can be used as an alternative to whole microorganisms. The proteins must be either purified from the source or produced by recombinant DNA technology. An advantage of protein antigens is that, similar to inactivated microorganisms, they can be broken down into multiple epitopes so no preselection or

screening of peptides is necessary (105). In addition, individuals can process the protein into epitopes that they can present on their MHC molecules. Unfortunately, protein antigens have many disadvantages. First, the protein must be expressed in a conformation that evokes an effective immune response and this may not be possible when producing the protein in bacteria. Both altered folding and glycosylation patterns can limit the ability of immune cells to recognize the antigen or to process and present it. However, purifying proteins from natural sources carries the risk of *in vivo* toxicities caused by contaminants. Second, protein antigens, in general, require more assistance to generate an immune response than whole microorganisms do (106). Third, protein antigens may be rapidly degraded by proteases *in vivo*, and, without protection, they will be too short-lived to elicit an immune response. Finally, although encapsulation of the protein in a delivery system will protect it against enzymatic degradation, the maintenance of the antigen's integrity during the preparation process is difficult. The protein is unstable during encapsulation, storage, hydration *in vivo*, and incubation at 37°C (3, 107). One successful recombinant protein vaccine has been developed, the prophylactic hepatitis B vaccine (108), however, this vaccine requires multiple immunizations to achieve effective immunity.

1.5.3 Peptide Antigen

Many of the disadvantages of traditional vaccines consisting of inactivated microorganisms can be avoided using peptide-based vaccines. Crucially, peptides are safer to use than traditional vaccines because they are not infectious in immunocompromised populations, such as the elderly or young children, and they cannot revert to an infectious form. In addition, peptides can be chemically synthesized, curtailing the use of biological systems for vaccine production (109). This allows for a safer vaccine that can be more readily approved for clinical use. From a manufacturing perspective, analysis, quality control, and the scale up of production are all straightforward (109). Peptide-based vaccines are chemically well defined

and easily synthesized (109). Furthermore, unlike vaccines based on recombinant proteins, correct protein folding is not an issue for peptide-based vaccines. From the immunological point of view, peptide-based vaccines can be designed to elicit an immune response against a select peptide or panel of peptides. This allows the immune response induced by the vaccine to be targeted to certain epitopes without distractions in the form of ineffective epitopes and can make it possible for the type of immune response to be controlled. In addition, peptides of the appropriate size and containing effective anchor residues can be used, ensuring effective antigen binding to MHC molecules. For these reasons, numerous peptide-based vaccines are at various levels of exploration from *in vitro* development to preclinical evaluation to clinical trials. Among the potential vaccine formulations currently undergoing clinical trial are a peptide from the V3 loop of the HIV virus (110), a synthetic peptide from the melanoma protein gp100 (111), a peptide from HER-2/neu for ovarian and breast cancer (112), and MUC1 mucin peptides for lung and breast cancer (113).

Despite the obvious advantages of peptide-based vaccines, challenges to the creation of highly effective vaccine formulations containing peptides remain. The two major disadvantages of peptide vaccines that must be surmounted are the low immunogenicity of the peptides and their short half-lives in the body. Both of these weaknesses can be overcome by encapsulation of the peptides in a delivery system such as liposomes or polymeric nanospheres. Unlike proteins, peptide antigens can be incorporated in delivery systems without concomitant loss of antigenicity (3, 107). A further disadvantage is that, because of the genetic variability in MHC molecules, not all people will necessarily be able to respond to a certain peptide. This can be addressed by careful selection of peptide antigens and delivery of multiple epitopes in one vaccine.

1.5.4 DNA

Immunization with plasmid DNA is emerging as an important method of vaccination. DNA immunization results in transfection of the host cells and expression of the encoded antigen. Since DNA can encode for multiple, entire proteins, numerous epitopes may be made available to the immune system. In addition, endogenous production of protein guarantees class I presentation of the antigen. Protein shed from the cells can be internalized, processed, and presented by APC. DNA vaccination is most effective if delivery of DNA directly to APC occurs because of the requirement for costimulation of the T cells. Currently there is a need for the development of non-toxic DNA delivery systems that can efficiently and selectively transfect APC such as DC.

1.6 Formulation

1.6.1 Particulate Antigen Delivery Systems

The rationale for particulate delivery systems is that they mimic microorganisms with respect to size and other characteristics, allowing for natural targeting of the vaccine to APC and optimal uptake by APC leading to endosomal and cytoplasmic delivery of antigen for processing and presentation on both class I and II MHC molecules. In addition, these antigen delivery systems can protect protein and peptide antigens from enzymatic degradation. Although the particulate delivery systems may possess some adjuvant activity, their predominate mechanism of immune enhancement is likely direct intracellular delivery of high concentrations of antigens to APC and co-delivery of adjuvant to the same cells. Particulate antigen delivery systems include liposomes, immunostimulating complexes (ISCOMS), nanospheres, and micelles, but this discussion will be limited to liposomes and PLGA nanospheres.

1.6.1.1 Liposomes

Liposomes are composed of concentric lipid bilayers that are separated by water compartments (2). When amphipathic lipids are exposed to an aqueous environment, interactions between the lipids and water lead to the spontaneous formation of closed bilayers (2). In the early 1960's, it was shown that a wide variety of molecules could be encapsulated in either the aqueous or the lipid layers of liposomes (2). Later, liposomes were recognized to have immunoadjuvant properties (114) and numerous animal immunization studies were performed (102, 115-117). Most recently, the first liposome-based vaccine, against hepatitis A, was licensed for use in humans.

Liposomes can differ considerably in size, from nanometric dimensions to the size of a living cell (2). The phospholipid composition also varies as does their organizational structure, from unilamellar to multilamellar vesicles (2). Depending on their physicochemical properties, compounds can be incorporated into either the aqueous compartment or the lipid bilayer (2). The properties of liposomes and their applications depend on the characteristics of the liposomal bilayer.

1.6.1.1.1 Composition

The principal lipid for the preparation of liposomes is usually a non-ionic lipid such as dipalmitoyl phosphatidyl choline (DPPC) (Figure 1-3A). The net surface charge of the liposomes can be altered by inclusion of charged lipids, such as the negatively charged dimyristoyl palmitoyl glycerol (DMPG) (Figure 1-3B). Combining two lipids has been shown to increase the stability of the liposomes (2). Stability of the liposomes and the minimization of leakage of liposomes contents can also be accomplished by the addition of cholesterol to the liposomal formulation (2). Cholesterol (Figure 1-3C), within the lipid bilayer, may regulate membrane fluidity and induce a condensing effect, which decreases membrane permeability and increases rigidity (118). It has also been shown that the effectiveness of a therapeutic liposomal vaccine for

adenocarcinomas depended on the presence of cholesterol in the lipid bilayer (1). Cholesterol may act to promote retention of the antigen within the liposomes for effective delivery to APC or it may directly influence the antigen uptake, processing and presentation by or the activation of APC.

1.6.1.1.2 Uptake and Processing by Cells

After subcutaneous (s.c.) injection into the body, a proportion of liposomes enter the lymphatic system and eventually the circulation where they can be cleared by the mononuclear phagocyte system (MPS). The majority of liposomes are retained at the site of injection where they can be phagocytosed by DC and carried back to the lymph node (2). Liposomes have the ability to deliver their contents by phagocytosis or by fusing directly with the cell membrane. This permits the encapsulated antigen to enter both the endogenous and the exogenous antigen processing pathways, activating both regulatory Th cells and the CTL that kill virally infected and cancerous self-cells.

1.6.1.1.3 Liposomes as an Antigen Delivery System

Liposomes are a viable vaccine delivery system for several reasons. In addition to being biodegradable and non-toxic, liposomes, as a particulate delivery system, are naturally targeted to APC. The size of the liposomes and their lipid composition can be tailored for optimal targeting to cells of the immune system (119-121). Liposomes can also deliver encapsulated antigen to both the endogenous and the exogenous antigen processing pathways (122). Moreover, liposomes can encapsulate hydrophilic, hydrophobic, or amphipathic materials including proteins, peptides, and DNA, and protect these materials from breaking down too quickly in the body (120). Finally, they can be used to co-encapsulate antigen with an adjuvant and may even themselves offer some adjuvant activity (123). In addition to increasing the immune-enhancing effects of the adjuvants, liposome-encapsulation ameliorates the toxic side effects of the adjuvants (102).

The primary disadvantage of liposomes is in their lack of stability. In terms of preparation, liposomal formulations can be prepared under relatively mild conditions, avoiding the use of organic solvents and lyophilization (124, 125). However, they can be physically unstable and aggregation and leakage of encapsulated compounds makes long-term storage challenging (124, 125). Moreover, large scale production of liposomes is arduous (126).

Liposome encapsulation augments the immunogenicity of poorly immunogenic subunit vaccines, eliciting both humoral and cell mediated immunity (115). The enhancement of humoral immunity by encapsulation of antigen into liposomes is due to the generation of a depot of antigen at the site of injection, prolonging the exposure of antigens to the immune system in the same way that alum does (2). After the encapsulation of antigen in liposomes, cell mediated immunity is improved by the targeting of antigen to APC and the co-delivery of adjuvant to these cells.

There has been considerable work performed in the area of formulating liposomes with specialized functions. A prominent area of investigation is the development of pH-sensitive liposomes. At a pH below 6.5, lipid bilayers containing dioleyl-phosphatidylethanolamine (DOPE) are destabilized and become fusogenic (127, 128). Using pH-sensitive and pH-insensitive liposomes, it has been possible to selectively trigger class I- or class II-restricted immune responses *in vitro* (129). After uptake of a DOPE-containing liposome into an endosome, the lowering of the pH causes the liposome to destabilize, fuse with the endosomal membrane, and release encapsulated antigen into the cytoplasm for processing through the class I MHC pathway. In contrast, after uptake, pH-insensitive liposomes are not destabilized by the acidic environment of the endosome and antigens are delivered to the lysosome, degraded, and presented in association with class II MHC molecules (2). However, *in vivo* no difference between the types of response elicited by the two types of liposomes could be observed (2). The ability of APC to process exogenous antigen, particularly in particulate form,

through the endogenous pathway for presentation on class I MHC likely renders the use of pH-sensitive liposomes unnecessary. As more information is uncovered about the mechanism of liposome immunoadjuvanticity and the effect of composition, site of antigen incorporation, and size on the immune response, liposomal formulations with specific tasks, such as preferential development of cell mediated over humoral responses could be devised.

1.6.1.2 Poly(D,L-lactic-co-glycolic acid) Nanospheres

Poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres have several features that make them an attractive vaccine delivery system. PLGA (Figure 1-4) is a biodegradable polymer currently approved for use in humans and is suitable for the formulation of recombinant proteins (130, 131), synthetic peptides (132, 133), and plasmid DNA (134). Delivery of antigen in PLGA nanospheres can induce humoral (130, 133), T helper (130, 133, 134), and cytotoxic T lymphocyte (135-138) responses *in vivo*. Finally, formulation characteristics can be tailored for optimal immune activation.

Encapsulation of peptide or protein antigen in PLGA nanospheres offers several challenges. During the preparation of nanospheres, the antigens are exposed to organic solvents and water-oil interfaces, and lyophilized; all procedures that are capable of denaturing antigen, a major issue for protein antigen in particular (139). During the degradation of PLGA nanospheres, antigen is exposed to a reduced pH, another potential source of antigen impairment (139). However, if the potency of the antigen can be maintained, PLGA nanospheres offer an advantage over liposomes in terms of stability during long-term storage (124, 125). Furthermore, the use of lyophilized vaccine formulations simplifies storage and distribution of vaccines.

1.6.1.2.1 Composition

PLGA nanospheres consist of antigen homogeneously dispersed in a matrix of polymer. PLGA is a biodegradable, biocompatible polyester approved for

human use (140, 141), consisting of lactic acid and glycolic acid linked together through ester bonds. Degradation of the polymer extracellularly involves random nonenzymatic hydrolysis of the ester bonds to liberate lactic and glycolic acid, which enter the normal metabolic pathways in the body. The rate of antigen release from PLGA nanospheres can be controlled by altering factors such as the lactic acid: glycolic acid ratio and the molecular weight of the polymer, both of which affect the degradation rate of the polymer (142-145). For example, PLGA with a 50:50 copolymer ratio displays more rapid degradation than PLGA with higher proportions of either monomer. The release of antigen from PLGA nanospheres is biphasic with an initial surface release followed by a lag phase of little or no release and then continuous release due to bulk erosion of the polymer (146, 147).

1.6.1.2.2 Uptake and Processing by Cells

The majority of investigations into the cellular uptake of PLGA nanospheres by APC have focused on Mϕ. Uptake of PLGA nanospheres by Mϕ has been demonstrated both *in vivo* and *in vitro* (130, 148, 149). This uptake is affected by size, surface charge, hydrophobicity and particle characteristics of PLGA nanospheres can be manipulated to increase phagocytosis. Due to the accumulating evidence that DC are the key APC, the ability of DC to phagocytose PLGA nanospheres is of increasing interest. Recently, the uptake of PLGA nanospheres by mouse DC *in vivo* has been demonstrated (150) but further studies are required to characterize this phagocytosis and elucidate the variables affecting this uptake.

After phagocytosis by APC, the PLGA nanospheres are degraded and their contents released in the endosome. Intracellular degradation is more complicated than extracellular degradation as it involves hydrolytic enzymes and the catalytic activity of the acidic endosomal/lysosomal compartments, in addition to random hydrolysis of backbone esters (151, 152). Similar to extracellular degradation, intracellular degradation is dependent on the bulk

properties of the polymer and this degradation can also be controlled by modifying polymer properties such as molecular weight (148, 149).

1.6.1.2.3 PLGA Nanospheres as an Antigen Delivery System

PLGA nanospheres can be used to incorporate a variety of different antigens for targeted delivery to APC, including DC. They can also co-encapsulate antigen and adjuvant for delivery to the same cell. Antigen delivered by PLGA nanospheres can be processed and presented via both the MHC class I and II pathways to stimulate both Th and CTL responses. PLGA nanospheres can also release antigen extracelluarly where it can bind to immunoglobulins on the surface of B lymphocytes and stimulate a humoral response.

The first investigations into the delivery of antigen using PLGA microspheres involved the use of protein encapsulated in microspheres for the induction of humoral responses (153-156). It has been shown that immunization with microspheres containing protein antigen induces systemic IgG responses comparable to those seen after immunization with CFA (157) or alum (155, 156). Furthermore, studies have demonstrated that peptide encapsulated in PLGA microspheres has the ability to elicit a humoral response as or more effective than the response induced by CFA (158).

Humoral immunity is primarily effective in situations involving external pathogens. In the case of cancer, or when an infection is caused by an intracellular pathogen, a cell mediated response is necessary. Investigations have demonstrated that both protein (154, 156, 159) and peptide (158) loaded PLGA nanospheres can elicit T cell responses comparable to those of control formulations containing alum. These proliferative responses are affected by the particle characteristics. In particular, it has been established that the T cell response was strongest for a rapid release formulation of low molecular weight PLGA (MW=8000) with a particle size of <10 μm in diameter compared to slower release formulations of high molecular weight PLGA (MW=60,000) with a particle size of >10 μm in diameter (158). The

difference in particle size, which has already been shown to affect the intensity of the immune response (142), makes it difficult to determine the outcome changing the molecular weight had on the T cell response. The effect of antigen-release kinetics on the induction of T cell responses requires further investigation.

It has been demonstrated that the type of response elicited by antigen is partially dependent on the mode of antigen delivery (133, 160, 161). It is possible that encapsulation of antigen into PLGA nanospheres could alter the type of immune response generated. The ability to manipulate the type of immune response developed after vaccination is a key reason why PLGA nanospheres are a potential delivery system for therapeutic vaccines.

1.7 Therapeutic Vaccines for Viral Diseases

1.7.1 Hepatitis B

The hepatitis B virus (HBV) is a noncytopathic, hepatotropic, enveloped virus that causes acute and chronic liver disease and hepatocellular carcinoma (162). The vast majority of acutely-infected adults recover completely from the disease and clear the virus. Approximately 5-10% of infected adults become persistently infected and develop chronic hepatitis (162). Neonatally transmitted HBV infection, however, is rarely cleared and greater than 90% of children infected with HBV become chronically infected (162). Despite the availability of a prophylactic vaccine against the HBV, there are 400 million carriers of the virus worldwide and there is no effective therapy available. Chronic infection with HBV is prevalent in many parts of Asia and can lead to cirrhosis and hepatocellular carcinoma (163).

1.7.1.1 Immune Response to Hepatitis B

The antibody response to the HBV envelope antigens is a T cell-dependent response (162). Because these anti-envelope antibodies are detected in patients who clear the disease and recover from acute hepatitis but are not

observed in patients who develop chronic infection, it is believed that they play an important role in clearing the HBV (162). Antibody can bind to free virus and prevent the entry of virus into susceptible cells while removing the virus from circulation. However, antibody alone is not effective for recovery from hepatitis B infection because a cell mediated response is required for the eradication of intracellular virus.

In patients with self-limited, acute hepatitis, a vigorous human leukocyte antigen (HLA) class-II restricted, $CD4^+$ Th response to multiple epitopes in hepatitis B core and surface antigens (HBcAg, HBsAg) is detected (162). Since this response is temporally associated with viral clearance in patients with acute hepatitis and since it is weak in patients who develop chronic hepatitis B, it is widely acknowledged that this class II-restricted response to HBcAg and HBsAg plays a critical role in viral clearance and disease recovery (162). This anti-viral $CD4^+$ response acts in several ways. First, the Th cells probably assist in the development of an effective CTL response to viral antigens since the timing and extent of the $CD4^+$ T cell response during acute hepatitis parallels the CTL response measured (162). Second, HBcAg-specific T cells likely provide help to B lymphocytes for the production of neutralizing antibodies (162). Without the establishment of a robust, antigen-specific Th response to proteins from the HBV, chronic disease ensues.

During chronic infection, the $CD4^+$ Th response to viral antigens is much less vigorous than in the case of acute infection (162). Both Th cells and CTL specific for the HBV are present but they are found at low frequency and exhibit low levels of activity (162, 164). Since viral persistence and the development of chronic disease and its sequelae are believed to be due to the lack of an appropriate response, activating a Th1-type immune response in patients may alter the clinical outcome of the HBV infection. A therapeutic vaccine that could accomplish this switch in the type of immune response could lead to disease resolution.

1.7.1.2 Hepatitis Core Antigen

Since it is apparent that a T cell response against epitopes from the core antigen of the HBV is required for viral clearance and recovery from disease, HBcAg is an important candidate in the design of a therapeutic vaccine for the treatment of chronic hepatitis B. A synthetic peptide containing a T helper epitope in mice, HBcAg residues 129-140, has been shown to generate a Th2-type response, based on cytokine profile and antibody isotype, in mice of the $H\text{-}2^b$ haplotype after immunization with a formulation of peptide emulsified with CFA (165). $HBcAg_{129\text{-}140}$ is a therapeutically relevant antigen that allows investigation into the role of formulation in modifying the response to the antigen.

1.8 Therapeutic Vaccines for Cancer

The immune surveillance theory states that as abnormal cells arise in the body, they are typically killed by cytotoxic T cells that respond to mutated self antigens on the surface of the cell (166, 167). Thus, the establishment of a cancerous tumour can be regarded as a failure of the immune system. Therapeutic vaccines seek to activate the body's own immune system to recognize and specifically kill the cancerous cells, leading to tumour regression with a minimal loss of healthy cells.

The development of therapeutic vaccines requires the identification of protein antigens that can evoke a tumour specific immune response. These antigens may be proteins that are expressed solely on tumour cells, proteins that are overexpressed on tumour cells, or proteins that are present on cancerous cells in a mutated form. Many tumour antigens, such as carcinoembryonic antigen and prostate-specific antigen, belong to a class of tumour antigens that are also expressed on normal epithelial cells and therefore might have induced immunological tolerance. Immunotherapy can overcome this tolerance to a self-antigen and activate these T cells.

The activation of T cells specific for tumour cells by immunotherapy may not result in tumour eradication. There are several possible mechanisms for failure. Firstly, tumours may downregulate class I MHC on their surface, limiting the recognition of tumour cells by CTL (168). In addition, the decreased MHC I expression may prevent the activation of cytotoxic pathways in the tumour cell after CTL binding (168). Tumour cells may also be resistant to apoptotic signals from CTL (168). Furthermore, tumours are highly effective at downregulating their expression of certain antigens so the selection of an appropriate antigen is critical (169).

1.8.1 MUC1

MUC1 mucin is a highly glycosylated membrane protein expressed on the apical surface of ductal epithelial cells (170-175). An underglycosylated form of MUC1 is highly expressed on adenocarcinomas of the pancreas, breast, ovary, prostate, and lung (176, 177). In addition to the mutations to the molecule itself, its expression is altered; on cancerous cells, MUC1 is located on the entire surface of the cell rather than simply the apical surface (178). The decreased glycosylation of MUC1 in cancer exposes an area of the protein that consists of tandem repeats of a 20 amino acid sequence (179). In addition, the lack of glycosylation alters the processing and presentation of mucin-derived peptides on Class I HLA molecules on the surface of cancerous cells (180). The expression of epitopes on cancerous cells that are not found on healthy epithelial cells and the presence of T cells that can recognize these epitopes, make MUC1 a potential target for immunotherapy for a variety of types of cancer.

1.8.1.1 MUC1 Lipopeptide

Situated in the tandem repeat region of the MUC1 mucin, a 25 amino acid peptide with a lipid moiety attached, BLP25, is a strong candidate for the immunotherapy of MUC1$^+$ cancers. Not only does BLP25 contain epitopes with the capacity to bind to several HLA class I molecules including HLA-A11, A3, A2.1, and A1, but BP24, the sequence of which is contained in BLP25,

also appears to be a permissive epitope for HLA class II-restricted CD4$^+$ T cell responses (181-183). In addition, it has been demonstrated in mice that a liposomal formulation of BLP25 can elicit a potent anti-MUC1 T cell response that is protective against tumour challenge (160, 161). However, this *in vivo* study does not closely enough mimic the therapeutic situation; in mice, MUC1 is a foreign antigen and the mice are naïve to it, while in a human cancer patient, MUC1 is a self-antigen and tolerance to the antigen may prevent a therapeutic response.

1.9 Research Proposal

The goal of this research was to investigate the modification of immune responses through antigen delivery approaches. Therapeutically relevant peptide antigens were encapsulated in liposomes and nanospheres and the immune response to them was characterized *in vivo* and *in vitro*. This series of investigations also examined the ability of antigen delivery systems to deliver antigen to human DC and stimulate a T cell response to an encapsulated peptide.

1.9.1 Hypotheses

1) The incorporation of a Th2-inducing peptide in liposomes along with MPLA will elicit a Th1-type response *in vivo*.
2) The incorporation of a Th2-inducing peptide in PLGA nanospheres along with MPLA will elicit a Th1-type response *in vivo*.
3) The incorporation of a Th2-inducing peptide in PLGA nanospheres will induce a Th1-type response even after the mice have been primed with a Th2-inducing formulation of the peptide.
4) Human DC can phagocytose PLGA nanospheres as readily as Mϕ.
5) After uptake of PLGA nanospheres containing a therapeutically relevant antigen, human DC can process the antigen, present it to T cells and stimulate a response.

1.9.2 Objectives

1) To formulate therapeutically relevant peptides in liposomes and nanospheres and characterize the formulations.
2) To investigate and characterize the immune responses against the formulations *in vivo* in naïve and primed mice.
3) To establish cultures of human DC and demonstrate and characterize the phagocytosis of PLGA nanospheres by DC.
4) To formulate a therapeutically relevant peptide in PLGA nanospheres and investigate the ability of human DC to process and present antigen to T cells and elicit a response.

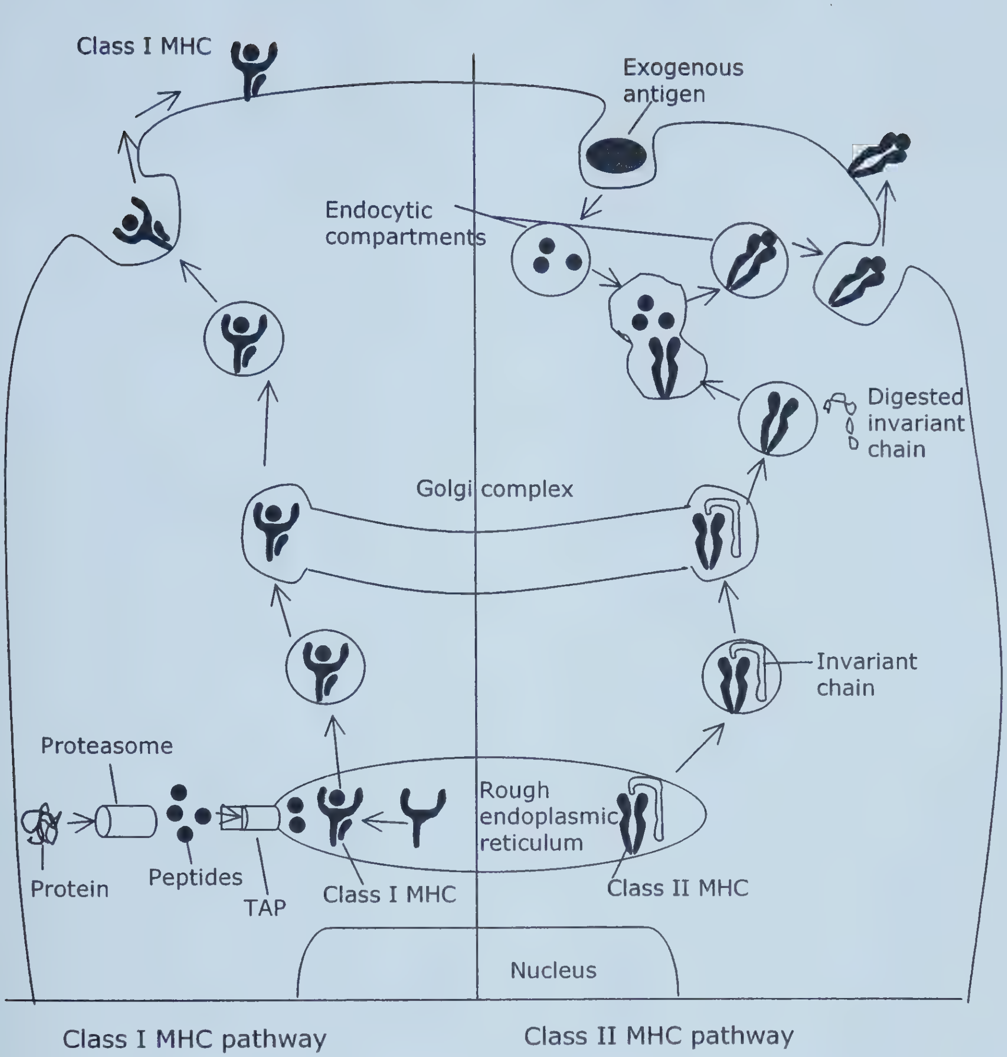

Class I MHC
Exogenous antigen
Endocytic compartments
Digested invariant chain
Golgi complex
Invariant chain
Proteasome
Rough endoplasmic reticulum
Protein
Peptides
TAP
Class I MHC
Class II MHC
Nucleus
Class I MHC pathway
Class II MHC pathway

Figure 1-1. MHC class I and MHC class II antigen presentation pathways.

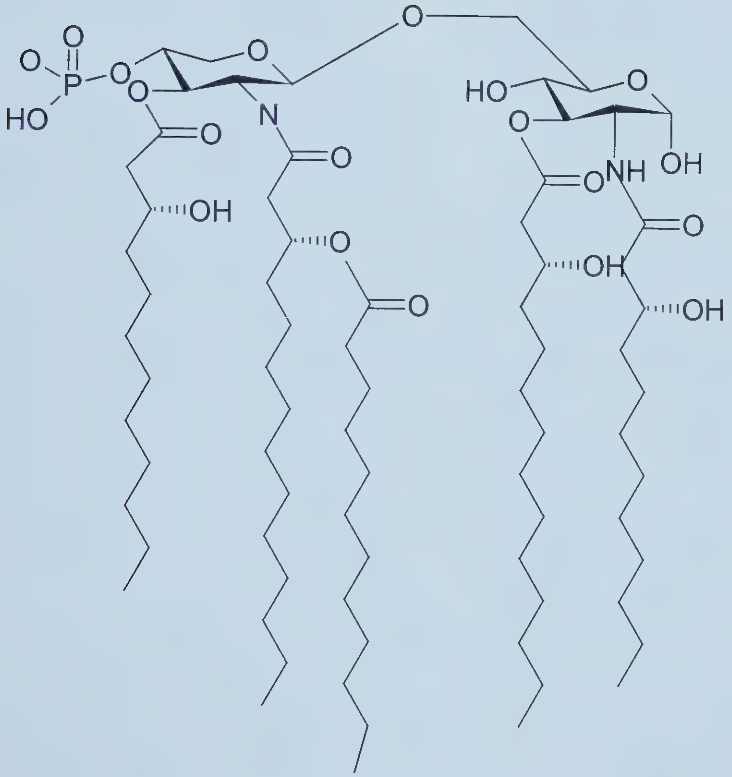
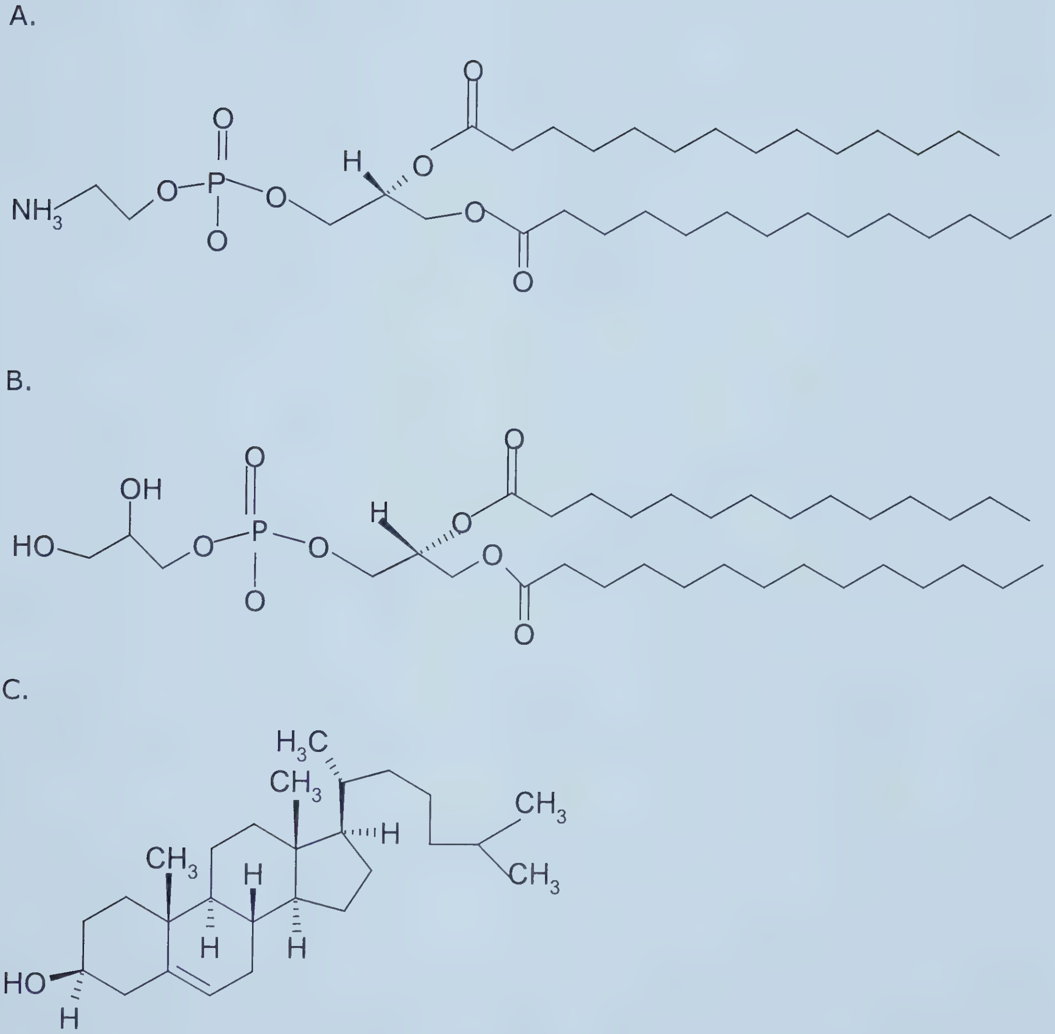


Figure 1-2. Chemical structure of MPLA.


A.
NH3
O
P
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H
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O
B.
OH
HO
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O
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H
O
O
O
O
C.
H3C
CH3
CH3
H
CH3
CH3
CH3
H
H
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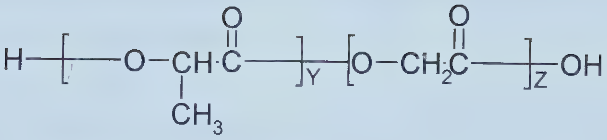
HO
H

Figure 1-3. Chemical structures of lipids. A. Dipalmitoyl phosphatidyl choline (DPPC). B. Dimyristoyl phosphatidyl glycerol (DMPG). C. Cholesterol



$$H\left[O-\underset{\displaystyle CH_3}{CH}-\overset{\displaystyle O}{\overset{\|}{C}}\right]_Y\left[O-CH_2-\overset{\displaystyle O}{\overset{\|}{C}}\right]_Z OH$$

Figure 1-4. Structure of poly(D,L-lactic-co-glycolic acid) (PLGA).

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Chapter Two

Characterization of Liposomal and Nanosphere Formulations of Therapeutically Relevant Peptides

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2.1 Introduction

Encapsulation of peptide antigens in liposomes or polymeric nanospheres protects the peptides from degradation and enhances their immunogenicity. Prior to use, vaccine formulations of encapsulated peptide must be fully characterized with respect to the physicochemical properties of the formulation including size, peptide content, and release characteristics.

Size is a critical factor for particulate antigen delivery systems. A particle size of <10 μm in diameter is required for potentiation of an immune response (1-3). Particles under 10 μm in diameter can be phagocytosed by antigen presenting cells and carried to the draining lymph node where the antigen is presented to T cells. In order to establish that the vaccine formulations can be taken up by antigen presenting cells, the sizes of peptide-containing liposomes and nanospheres were characterized.

Methods of peptide encapsulation vary highly with respect to encapsulation efficiency (EE) and the peptide dose can vary considerably depending on the formulation. The dose of the peptide antigen can significantly influence the type and magnitude of the immune response, which may have detrimental or beneficial effects in several disease states (4, 5). Therefore, the development of methods that permit rapid and accurate determination of peptide content in particulate formulations is important. Such methods are also essential for scale up, quality control, and determination of batch-to-batch consistency of the formulations.

Quantification of encapsulated substance may be done either without disruption of the particles or after extraction of the substance from the formulation. Without extracting the material from the formulation, the EE may be determined based on the amount of unencapsulated molecule in the supernatant following centrifugation of the formulation (6-12) or after passage over a gel filtration column (13-18). However, this indirect method of measurement may lead to inaccurate estimations for peptide content due to loss of peptide from degradation or from binding with

container surfaces. Alternatively, after separation of free from encapsulated material, direct estimation of the encapsulated compound in the formulation may be performed based on its fluorescence properties (6, 17) or radioactivity (9, 13, 18, 19). However, if fluorescence or radioactivity is introduced into the compound by significant chemical modification, the encapsulation efficiency of the modified compound may vary significantly from that of the native molecule of interest. In addition, the labeling process can be time consuming and expensive and must be redone for each additional molecule to be investigated.

A more effective method involves extracting the substance to be measured from the formulation and directly determining the amount of compound through one of several methods. Substances can be extracted from liposomes by many extraction media; Triton-X (6, 10, 14-17), chloroform (19), methanol (20), sodium chlorate (8), 1% octyl-β-glucoside (8), SDS (11), and acidified isopropyl alcohol (12) have all been used. Several methods have been used to extract substances from poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres: dichloromethane/water (2, 21-23), alkaline hydrolysis/sodium dodecylsulfate (24-26), dimethyl sulfoxide/water (27), and chloroform/water (28). Once the encapsulated compound is separated from the lipids or polymer, the amount of compound can be measured in any of several ways: measurement of activity of the compound (14-16), spectrophotometry (8, 11, 12, 18), radiation counting (18, 19), weight of substance recovered (20), or reverse phase (RP) HPLC (10, 12).

Many of these methods are wholly ineffective for determining the amount of therapeutic peptide present. In contrast, RP-HPLC is the standard method for quantification of peptides and is favoured for the determination of peptide encapsulation efficiency in vaccine formulations for several reasons. First, RP-HPLC allows the determination of peptide content without interference by lipids or polymer. In addition, the UV absorption spectra of the encapsulated peptide and unencapsulated peptide can be compared to ensure that the peptide has not been altered

by encapsulation or extraction. Finally, RP-HPLC is an easy and replicable method and is suitable for gauging relatively small amounts of peptide. However, in order to use the RP-HPLC method to determine the amount of peptide inside liposomes or nanospheres, the peptide must be extracted from the formulations with a substance that will not modify the peptide, that will remove the majority of lipids or polymer from the sample, and that is compatible with the RP-HPLC and its solvents.

The practice of extracting therapeutic substances from liposomes and nanospheres in order to determine the amount of substance encapsulated is highly effective but it has not been adequately explored in the context of therapeutic peptides. Similarly, the use of RP-HPLC to determine peptide quantity is a standard procedure that has yet to be carefully examined in the framework of liposomal and polymeric formulations. The purpose of this series of investigations was to determine how best to extract a variety of peptides and lipopeptides from vaccine formulations in order to examine encapsulation efficiency using an RP-HPLC.

In addition to the size and peptide content of the delivery systems, the retention and release characteristics of the formulation must be characterized. In contrast to the aims of a number of vaccine formulations, the goal of the formulations used in this series of investigations is not slow release of the antigen over long periods or pulsed release of repeated booster doses of antigen at spaced intervals. It is desired that these formulations retain the peptide with minimal release at the site of injection. Following phagocytosis of the particles by antigen presenting cells, release of the peptide antigen into the Class I and Class II presenting pathways is desired. The amount of peptide released from liposomes and PLGA nanospheres *in vitro* over 24 hours was examined in order to confirm that the antigen was retained for eventual delivery to antigen presenting cells.

2.2 Materials and Methods

2.2.1 Materials

A panel of peptides of varying hydrophobicity/hydrophilicity characteristics, prepared by solid-phase synthesis, from MUC1 mucin, hepatitis B virus core antigen (HBcAg), and collagen IV were used. Peptide amino acid sequences are reported in Table 2-1. Average hydrophobicity (AH) is the sum of all hydrophobicity values (29) for the given sequence divided by the sequence length (Table 2-2). The higher the AH value, the more hydrophobic the molecule. AH values were calculated using PepTools Version 2.0 (BioTools Inc., Edmonton, AB, Canada). Peptides from the hepatitis B core antigen were synthesized by Dr. David Wishart's laboratory, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (Edmonton, AB, Canada). Biomira Inc. (Edmonton, AB, Canada) supplied all other peptides.

The lipids used were dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylglycerol (DMPG) (Genzyme Pharmaceuticals, Cambridge, MA, USA), and cholesterol (CHOL) (Sigma Chemical Co., St. Louis, MO, USA). PLGA (lactic acid: glycolic acid ratio 50:50; BPI, Birmingham, AL, USA) with molecular weights of 6000 and 50,000 was used for formulation of the nanospheres.

2.2.2 Preparation of Formulations

2.2.2.1 Liposomes

Liposomes were prepared using a modified freeze-thaw method (30). Briefly, lipids were used at a molar ratio of 3 DPPC: 1 CHOL: 0.25 DMPG. Lipids in solution were coated onto round-bottom flasks using a rotary evaporator (Buchi RE111 Rotavapor, Buchi Laboratiums-Technik, Switzerland). The volumes used were 1.95 ml of DPPC (16 mg/ml) in $CHCl_3$, 1.8 ml of CHOL (3.08 mg/ml) in $CHCl_3$, and 2.2 ml of DMPG (1.1 mg/ml) in a mixture of methanol and $CHCl_3$ (1:3). The flasks were incubated in a 43°C vacuum oven overnight to remove residual solvent. For empty liposomes, the lipids were rehydrated with 2 ml of PBS. For

liposomes containing peptide, lipids were rehydrated with 2 ml of PBS containing 300 μg of the appropriate peptide. Flasks were incubated in a 53°C water bath and then vortexed until lipids were removed from the sides of the flasks and the solution appeared homogenous. Five cycles of the following were performed: freeze flasks in dry ice/acetone, thaw for 40 minutes at room temperature, incubate at 41°C in water bath for 5 min, vortex for 30 s. Liposomes were collected via ultracentrifugation (150,000 x *g* for 20 minutes) (Model LB-55, Beckman Instruments Inc., Mississauga, ON, Canada), and washed twice with PBS. Supernatants (S1 and S2) were collected after each centrifugation for analysis of peptide content. The liposome pellet was resuspended in a replacement volume of PBS.

2.2.2.2 PLGA Nanospheres

PLGA nanospheres containing $HBcAg_{129-140}$ were prepared using a water/oil/water solvent evaporation technique while nanospheres containing BLP25 were prepared using a single emulsion solvent evaporation technique (23). For $HBcAg_{129-140}$ nanospheres, 100 μl of a 2 mg/ml solution of $HBcAg_{129-140}$ was emulsified in 500 μl of chloroform (Fisher Scientific, Nepean, ON, Canada) containing 100 mg of PLGA polymer (either 6000 MW or 50,000 MW) using a microtip sonicator (Model XL2010, Heat Systems INC., Farmingdale, NY, USA). The resulting primary emulsion was added into 2 ml of 9% w/v polyvinyl alcohol (PVA) (87-89% hydrolyzed, MW 31-50,000; Aldrich Chemical Co. Inc., Milwaukee, WI, USA) and was further sonicated to form the secondary emulsion. For nanospheres containing BLP25, 100 μL of a 1 mg/ml solution of BLP25 was added to 400 μl of chloroform containing 100 mg of PLGA. The BLP25/PLGA solution was emulsified with 2 ml of 9% w/v polyvinyl alcohol. Both the $HBcAg_{129-140}$ emulsion and the BLP25 emulsion were added drop wise into 8 ml of 9% w/v PVA and stirred for 3 h to evaporate the chloroform. The nanospheres were then collected by ultracentrifugation (40,000 x *g*, 10 minutes at 20°C), washed twice with distilled water (20 ml) to remove residual PVA, resuspended in 5 ml

distilled water, and freeze-dried for 3 days. Nanospheres were stored at –20°C until needed.

For the peptide recovery experiments, a batch of empty nanospheres was prepared by each method. For single emulsion nanospheres, 100 μl of chloroform replaced the peptide solution. For double emulsion nanospheres, 100 μl of PBS replaced the peptide solution.

2.2.3 Particle Sizing

Particle sizes of liposomes and nanospheres were determined by dynamic light scattering (model BI-90 Particle Sizer, Brookhaven Instruments Corp., Holtsville, NY). The samples were diluted 100x with PBS, and size was measured at 25°C.

2.2.4 RP-HPLC Analysis

Samples for the analysis of peptide recovery from liposomes and encapsulation efficiency of liposomes were manually injected into a Waters 625LC HPLC (Waters, Mississauga, ON, Canada). Samples for analysis of nanospheres and for release studies of both nanospheres and liposomal formulations were injected into the RP-HPLC using a Gilson 234 Autoinjector (Mandel Scientific Co., Guelph, ON, Canada). The RP-HPLC was equipped with a C_{18} reverse phase column (8 x 100 mm) and an UV detector (Waters 486) set at 210 nm. The mobile phases employed were A [10% acetonitrile (Fisher Scientific) and 0.1% trifluoroacetic acid (Sigma, St. Louis, MO, USA) in water] and B (70% acetonitrile and 0.085% trifluoroacetic acid in water). Elution conditions for each peptide are outlined in Table 2-3. The peptide concentration in all samples was determined by comparing the UV absorbance of the peptide with a standard curve (R^2 >0.998) generated under the same conditions.

2.2.5 Peptide Recovery

2.2.5.1 Liposomes

All experiments were set up in triplicate in microcentrifuge tubes. To each tube was added 150 μl of empty liposomes. Liposomes were spun down

at 10,300 x *g* for 12 minutes and all supernatant was removed. To the pellets, 40 μg of the appropriate peptide was added in 20 μl of PBS. Tubes were vortexed to thoroughly mix the peptides with liposomes. To each tube was added 200 μl of methanol, 200 μl of 98% ethanol, or 200 μl 25% ethanol (ethanol diluted in water). The tubes were vortexed until the lipid pellets broke up. Samples were then incubated in a 51°C water bath for 30-60 minutes. After removal from the water bath, tubes were centrifuged at 10,300 x *g* for 12-15 minutes. The supernatant was analyzed for peptide content by RP-HPLC; two injections of 50 μl each were made to the RP-HPLC. For each peptide/extraction media combination there were six injections, 2 each from the 3 tubes. The amount of peptide in each injection was calculated using a standard curve. Standard curves for all peptides used in this set of investigations had R^2 values of >0.998. The 6 quantities were averaged and compared to the amount of peptide added to the liposomes.

(1) $\%_{REC} = P_{REC}/P_{ADD} \quad X\ 100$

Where $\%_{REC}$ is the percent recovery, P_{REC} is the peptide recovered after mixing with liposomes, and P_{ADD} is the amount of peptide added to empty liposomes.

2.2.5.2 Nanospheres

All experiments were set up in duplicate in microcentrifuge tubes. To 10 mg of empty nanospheres was added 12.5 μl of a 2 mg/ml solution of either $HBcAg_{129-140}$ or BLP25. The mixtures were vortexed to thoroughly mix the peptide with the nanospheres.

2.2.5.2.1 Tetrahydrofuran (THF) Method

To each tube was added 500 μl of THF (Caledon Laboratories Ltd., Georgetown, ON, Canada) and the tubes were shaken by hand until the contents became clear. Prior to centrifuging the tubes at 10,300 x *g* for 10 minutes, 500 μl of water was added to each tube and the tubes were vortexed briefly. Two 200 μl samples of the supernatant from each tube were analyzed by RP-HPLC.

(2) $\%_{REC} = P_{REC}/P_{ADD} \times 100$

Where $\%_{REC}$ is the percent recovery, P_{REC} is the amount of peptide recovered after mixing with nanospheres, and P_{ADD} is the amount of peptide added to empty nanospheres.

2.2.5.2.2 Acetonitrile Method

One ml of acetonitrile (Caledon Laboratories Ltd., Georgetown, ON, Canada) was added to each tube. After incubation at room temperature for 15 minutes, the samples were vortexed. Tubes were centrifuged at 10,300 x *g* for 10 minutes and the supernatant was discarded. The pellet was resuspended in 500 µl of water and two samples of 200 µl were analyzed by RP-HPLC.

(3) $\%_{REC} = P_{REC}/P_{ADD} \times 100$

Where $\%_{REC}$ is the percent recovery, P_{REC} is the amount of peptide recovered after mixing with nanospheres, and P_{ADD} is the amount of peptide added to empty nanospheres.

2.2.6 Encapsulation Efficiency

2.2.6.1 Liposomes

All experiments were set up in triplicate in microcentrifuge tubes. Samples of liposome suspensions (150 µl) were added to microcentrifuge tubes, centrifuged, and 100 µl of supernatant (S3) was removed from each tube. To the liposome pellets was added 150 µl of the extraction media that proved to give the best recovery for the peptide in the peptide recovery experiments. The tubes were vortexed to break up the liposome pellet and incubated in a 53°C water bath for 30 minutes. Tubes were centrifuged at 10,300 x *g* for 12-15 minutes. Two injections of 50 µl aliquots each of supernatant (L for liposomes) were made to the RP-HPLC to determine the amount of peptide encapsulated in liposomes. Fifty microliters each of a 1:3 aqueous dilution of S1 (the first supernatant removed during the preparation of the liposomes) and undiluted S2 (the second supernatant removed during the preparation of the liposomes) and

S3 was injected into the RP-HPLC. The quantity of peptide in the injected volumes was calculated by comparison with a standard curve ($R^2 > 0.998$) for the peptide. The quantity of the peptide in supernatants S1, S2, and S3 were calculated taking into account the dilution factor and the total volume of each supernatant. The total amount of peptide encapsulated in liposomes (L_{total}) was calculated based on the value of peptide in L, the total volume of liposomal batch, and the % recovery efficiency of the extraction procedure. The EE and the total amount of peptide accounted for in the analysis were calculated using equations 4 and 5

(4) $EE = L_{TOTAL}/P_{TOTAL} \times 100$

Where EE is the encapsulation efficiency of the peptide in liposomes, L_{TOTAL} is the total amount of peptide in liposomes, and P_{TOTAL} is the total amount of the peptide used for formulation.

(5) $\%_{ACC} = (S1 + S2 + L_{total})/ P_{TOTAL} \times 100$

Where $\%_{ACC}$ is the percentage of peptide accounted for, S1 is the amount of peptide in the first wash, S2 is the amount of peptide in the second wash, L_{total} is the total amount of peptide in the liposomes, and P_{TOTAL} is the total amount of peptide used for formulation.

2.2.6.2 Nanospheres

2.2.6.2.1 THF Method

All experiments were performed in duplicate in microcentrifuge tubes. To each tube was added between 6 and 7 mg of nanospheres and 500 μl of THF and the tubes were shaken by hand until the contents became clear. Prior to centrifuging the tubes at 10,300 x *g* for 10 minutes, 500 μl of water was added to each tube and the tubes were vortexed briefly. Two 200 μl samples of the supernatant from each tube were analyzed by RP-HPLC. The amount of peptide in the injection volume was determined by comparison with a standard curve ($R^2 > 0.998$). The amount of peptide in the nanosphere sample was calculated using the volume of the extraction supernatant. The quantity of peptide in the batch of nanospheres was calculated using the weight of the batch.

(6) $EE = L_{TOTAL}/P_{TOTAL} \times 100$

Where EE is the encapsulation efficiency of the peptide in nanospheres, L_{TOTAL} is the total amount of peptide in nanospheres, and P_{TOTAL} is the total amount of the peptide used for formulation.

2.2.6.2 Acetonitrile Method

All experiments were performed in duplicate in microcentrifuge tubes. To each tube was added between 6 and 7 mg of nanospheres and 1 ml of acetonitrile was added to each tube. After sitting at room temperature for 15 minutes, the samples were vortexed. Tubes were centrifuged at 10,300 x *g* for 10 minutes and the supernatant was discarded. The pellet was resuspended in 500 μl of water and two samples of 200 μl were analyzed by RP-HPLC. The amount of peptide in the injection volume was determined by comparison with a standard curve (R^2 >0.998). The amount of peptide in the nanosphere sample was calculated using the volume of the extraction supernatant. The quantity of peptide in the batch of nanospheres was calculated using the weight of the batch.

(7) $EE = L_{TOTAL}/P_{TOTAL} \times 100$

Where EE is the encapsulation efficiency of the peptide in nanospheres, L_{TOTAL} is the total amount of peptide in nanospheres, and P_{TOTAL} is the total amount of the peptide used for formulation.

2.2.7 Release over 24 Hours

Samples of liposomes (350 μl) and nanospheres (10 mg in 500 μl PBS) were incubated in a 37°C water bath for 24 hours. After incubation, samples were centrifuged at 10,300 x *g* for 15 minutes. For liposomes, two samples of 150 μl each were analyzed RP-HPLC. For nanospheres, two samples of 200 μl each were analyzed. Two hundred and fifty microliters of 25% ethanol was added to the liposome pellets remaining in the microcentrifuge tubes. The extraction procedure was then followed as described for the analysis of encapsulation efficiency. Either THF or

acetonitrile was added to nanosphere pellets and the extraction procedure described above was followed.

2.3 Results

2.3.1 Particle Size of Liposomes and PLGA Nanospheres

Particle sizes were measured for the different formulations using dynamic light scattering. Data is presented as the average +/- the standard deviation for samples from at least three different batches of each formulation (Table 2-4).

2.3.2 Peptide Recovery

2.3.2.1 Liposomes

Preliminary experiments confirmed the solubility of the peptides used in the candidate solvents. In order to select an appropriate extraction medium, the efficiency of the extraction media for peptide recovery from liposomal formulations was determined. For this purpose, peptide-free liposomal suspensions were spiked with a known amount of peptide and the peptide was extracted using different solvents and quantified by RP-HPLC. Comparison of the peptide recoveries allowed the evaluation of the three extraction media and the selection of the optimal solvent for determination of the encapsulation efficiency of each peptide. It also supplied a recovery value, which was used as a correction factor in calculating the encapsulation efficiency.

For each solvent, there was a wide range in the recovery values seen with the different peptides (Table 2-5). For 25% ethanol, the values ranged from 10% for the lipopeptide, BLP25, to 113 % for BP1-072, a collagen IV peptide. For 98% ethanol, values were seen from 20% for BP16 to 88% for the shortest HBcAg peptide. Recovery values for extractions using methanol ranged from 45% for BP16 to 87% for BP1- 072. For four peptides, BP16, BP24, BP1- 072, and BP25, recovery was clearly greatest with 25% ethanol followed by recovery with methanol and then 98% ethanol.

BP25 and BLP25 have the same amino acid sequence, differing only in the addition of a lipid moiety. Not surprisingly, this lipid moiety caused a difference in the effectiveness of recovery by different solvents. The sole lipopeptide in the experiment, BLP25, was most effectively recovered using 98% ethanol, although methanol was a close second. BLP25 was extremely poorly recovered using 25% ethanol.

For the longest hepatitis B peptide, $HBcAg_{120-140}$, recovery was greatest with 98% ethanol, and there was little difference between methanol and 25% ethanol. There were only small differences seen between the three solvents in the recovery of $HBcAg_{126-140}$ but 98% ethanol appears to be the best solvent. $HBcAg_{129-140}$ showed a different pattern than the other two peptides from the core antigen. The best solvent for recovery of the shortest HBcAg peptide was 25% ethanol, followed by 98% ethanol, with methanol being the least useful solvent.

2.3.2.2 Nanospheres

Peptide recovery from PLGA nanospheres was examined for two peptides and two polymer molecular weights. Preliminary experiments confirmed the solubility of the peptides in the candidate solvents. As for the analysis of peptide recovery from liposomes, empty formulations were spiked with a known amount of peptide. Following thorough mixing, two extraction procedures were tested: THF extraction and acetonitrile extraction. For THF extraction, nanospheres were dissolved in THF and water was added to precipitate out the polymer. Centrifugation pelleted the majority of the polymer and the supernatant was analyzed for peptide content by RP-HPLC. For extraction using acetonitrile, nanospheres were dissolved in acetonitrile. The polymer dissolved while the peptide remained suspended. The samples were spun down to pellet the peptide and the supernatant containing the polymer was removed. Water was added to dissolve the peptide and samples were analyzed by RP-HPLC. Results for BLP25 formulated in low molecular weight PLGA and $HBcAg_{129-140}$ formulated in low and high molecular weight polymer are in Table 2-6.

For all formulations studied, the acetonitrile method proved to be more effective for peptide recovery. The difference in recovery between the two methods was less for BLP25, 65% versus 87%, than for $HBcAg_{129-140}$ in either formulation, 47% or 50% versus 93% or 91%. There was no difference seen for $HBcAg_{129-140}$ recovered between formulations using low molecular weight PLGA and formulations using high molecular weight PLGA.

2.3.3 Encapsulation Efficiency

2.3.3.1 Liposomes

Each peptide was encapsulated into liposomes using the freeze thaw method, commonly used for encapsulation of hydrophilic molecules. For each peptide, the extraction medium that gave the highest recovery in the recovery experiment was used to extract the peptide from the liposomes for the encapsulation experiment.

Encapsulation efficiencies of the different peptides (Table 2-7) ranged considerably. BP1-072, a collagen peptide, gave the lowest encapsulation efficiency at 3% while virtually all (99%) of the lipopeptide, BLP25, was encapsulated inside the liposomes. The peptide containing an amino acid sequence identical to that of BLP25 but lacking the lipid moiety, BP25, had an EE of only 8%. The addition of the lipid moiety to the molecule greatly enhanced the level of encapsulation of the peptide.

The total amount of peptide accounted for was considerably less than 100% in most cases. Whether this was due to peptide loss during the preparation of the liposomes or due to inefficiencies in the extraction of the peptide from the liposomes remains to be seen.

2.3.3.2 Nanospheres

BLP25 and $HBcAg_{129-140}$ were encapsulated into nanospheres of either low or high molecular weight PLGA using a solvent evaporation technique. Both extraction methods were used to determine the encapsulation

efficiency of the peptide in the nanospheres. The peptide quantities were corrected using the recovery values from Table 2-6.

BLP25 was encapsulated efficiently in the formulation (Table 2-8). $HBcAg_{129-140}$ was encapsulated to a much lesser extent. Only 13% of the peptide was encapsulated in the low molecular weight formulation while 11% of the peptide was encapsulated in the formulation prepared with the higher molecular weight polymer (Table 2-8). The values for $HBcAg_{129-140}$ and BLP25 quantities after THF extraction were extremely low and inconsistent (data not shown) and it is believed that those numbers are not reliable.

2.3.4 Release of Peptide over 24 Hours

After immunization, it is important that peptide be retained in the formulation until phagocytosis by APC. The purpose of this study was to measure the release of peptide from formulations over 24 hours at 37°C to determine if a significant portion of peptide could be retained within the delivery systems. In addition to measuring the quantity of peptide released, the amount of peptide retained in the formulations was determined and the total peptide accounted for was calculated.

Liposomes containing $HBcAg_{129-140}$ were incubated at 37°C in a water bath. After 24 hours, the sample was centrifuged and the supernatant was analyzed by RP-HPLC. The liposome pellet underwent the extraction method as described and the amount of peptide retained in the liposomes was determined. No $HBcAg_{129-140}$ was detected in the supernatant after 24 hours. After ethanol extraction, 92% of the peptide was recovered from the liposomes. There was no substantial release of peptide from the liposomes after 24 hours of incubation and almost all of the peptide was recovered from the formulation (Table 2-9).

Prior to placement in a water bath, nanospheres were resuspended in PBS. After 24 hours, samples were centrifuged and the supernatant analyzed by RP-HPLC. Retained peptide was extracted from the nanosphere pellet by THF and acetonitrile extraction. The values for

peptide quantity after THF extraction were highly inconsistent (data not shown). Neither BLP25 nor HBcAg$_{129-140}$ was detected in any supernatant from any formulation after 24 hours. For all PLGA nanosphere formulations of HBcAg$_{129-140}$, close to or greater than 90% of peptide was retained in the pellet and recovered. There was no substantial release of either peptide from the PLGA nanospheres after 24 hours of incubation and most of the peptide was retained in the nanospheres (Table 2-9).

2.4 Discussion

As the number of peptide-based vaccines under investigation escalates, the development of methods to characterize peptide formulations becomes crucial. In addition to investigating the type and extent of immune response elicited by vaccine formulations and the behaviour of the liposomes and nanospheres *in vivo*, the physicochemical characteristics of the formulation must be examined, including particle size, quantity of antigen encapsulation and release of peptide.

Sizes of the different formulations varied somewhat. The liposomes containing HBcAg$_{129-140}$ had the largest average diameter, followed by nanospheres made by the double (w/o/w) emulsion technique while the nanospheres made by single emulsion (w/o) had the smallest particle size. There were no significant differences seen in particle size for the nanospheres made of PLGA with differing molecular weights. All formulations were of small particle size suitable for phagocytosis by APC. Since particles were in the nanometer size range, it is anticipated that antigen release would not be delayed to any great extent after uptake by APC. Preparation of PLGA nanospheres by the single emulsion method is most suited for achieving the lowest particle size. However, this requires solubilization of the antigen in organic solvents along with the polymer. This is easily achieved for lipopeptides such as BLP25, but may be rather difficult to achieve for highly hydrophilic peptides.

Of the potential methods to determine the EE of a peptide in particulate delivery systems, the extraction of the peptide from the formulations

using an extraction solvent followed by analysis of peptide content using an RP-HPLC is the most advantageous for several reasons. Firstly, it is replicable and easy to use. Secondly, it does not require chemical modification of the peptide. Finally, it can be used to analyze relatively small amounts of peptide.

Prior to using the RP-HPLC to determine the amount of peptide encapsulated inside liposomes, the peptide must be extracted from the liposomes using a solvent. The ideal solvent will be relatively inexpensive, readily available, non-toxic, and compatible with the RP-HPLC and its solutions. Many different solvents have been utilized for this purpose but to date there has been no systematic study of which solvents are most effective for the extraction of peptides from liposomes prior to analysis of peptide content using the RP-HPLC.

In order to examine the ability of several solvents to recover peptides from liposomes, empty liposomes were spiked with a known amount of peptide and three different extraction procedures were followed to determine the recovery of eight different peptides. The results demonstrated that the amount of peptide recovered depended on the combination of extraction medium and peptide. In particular, the optimal extraction medium depended on the chemical nature of the peptide being extracted. For the more hydrophilic peptides, $HBcAg_{129-140}$, BP16, BP24, and BP25, 25% ethanol proved to be the most effective extraction medium. For the more hydrophobic peptides, BLP25 and $HBcAg_{120-140}$, 98% ethanol was the most valuable solvent.

This investigation demonstrates that the best solvent for extraction of peptide from liposomes varies, depending on the nature of the peptide being examined. It may be possible to predict the optimal solvent by comparing the amino acid sequences of an untested peptide to tested peptides or by using software capable of predicting the average hydrophobicity of a peptide, however, this can only serve as a general guideline for the selection of the extraction media. The true suitability of

the solvent should be verified experimentally by determining peptide recovery from the formulation. Furthermore, in addition to the chemical nature of the peptide, the nature of the liposomal lipids may also significantly influence the optimal choice of the extraction medium. This requires further investigation.

Two solvent extraction techniques were investigated for the extraction of peptides from PLGA nanospheres, the THF and acetonitrile methods. Both $HBcAg_{129-140}$ and BLP25 were more effectively recovered when the acetonitrile method was used. Although the recovery of only two peptides was examined, the peptides differ considerably in their average hydrophobicity values; BLP25 with its lipid moiety is highly hydrophobic while $HBcAg_{129-140}$ is quite hydrophilic. The optimal solvent of choice for peptide recovery from liposomes was affected by the hydrophobicity levels of the peptide. It is possible that this is less of an issue for separation of peptide from nanospheres. Perhaps, with nanospheres, the nature of the polymer plays a more significant role in determining the solvent choice than does the nature of the peptide. Further work exploring this theory is needed.

Once the extraction medium with the highest recovery for each peptide from liposomes was identified, the EE was ascertained for each of the eight peptides in liposomes. The results established that there was a wide range of EE for the peptides we examined. It is known that the nature of the molecule affects the incorporation of the molecule into liposomes (31) and the results from these investigations supported this. The lipopeptide gave the highest EE; its lipid moiety bestowed an amphipathic nature to the molecule that allowed for an easier encapsulation. For some of the peptides we examined, such as BP1-072, which was encapsulated at about 2% efficiency, chemical modification or encapsulation by alternate methods may be necessary to increase the EE without decreasing the antigenicity of the molecule. Interestingly, there was no correlation found between the average hydrophobicity values and the amount of peptide encapsulated inside the liposomes. It is likely that both the amino acid

sequence and the distribution of hydrophilic and hydrophobic residues in the molecule play more significant roles in determining the EE of the peptides than the average hydrophobicity does.

For analysis of peptide EE in PLGA nanospheres, both the THF and acetonitrile methods were used and quantities were corrected using the established recovery values, however, area under the curve values for $HBcAg_{129-140}$ were so low with this method that it was determined to be unreliable. BLP25 was encapsulated at very high efficiency in the nanospheres while $HBcAg_{129-140}$ was encapsulated at only about 10%. Since the two peptides differ considerably in their hydrophobicity levels, this difference could be due to the nature of the molecules; however, the nanospheres were also prepared using slightly different procedures. Since it has been shown that when BLP25 is encapsulated in nanospheres using the w/o/w emulsion technique, the EE is 12% (32), it appears as if the method of incorporation may have a decided effect on the encapsulation efficiency of the peptide in PLGA nanospheres.

In addition to the size and peptide content of the vaccine formulations, the retention and release of the antigen from the delivery system must be characterized. For the purpose of immunotherapy designed to elicit a T cell response, formulations should retain the peptide with minimal release at the site of injection. Upon phagocytosis of the particles by antigen presenting cells, release of the peptide antigen into the Class I and Class II presenting pathways is desired.

For both liposomal and nanosphere formulations of BLP25 and $HBcAg_{129-140}$, no peptide was detected in the supernatant after incubation at 37°C for 24 hours. Analysis of the quantity of peptide retained in the formulations demonstrated that close to or greater than 90% of the peptide was retained for 24 hours. The data indicates that the formulations will not release antigen at the site of injection but will retain it for delivery to the Class I and II presenting pathways in APC.

The peptides used in this study were selected due to their significance in the development of therapeutic vaccines. MUC1 mucin is a very important cancer antigen expressed by most carcinomas including breast, ovarian, prostate, lung, head and neck cancer and is a major target for therapeutic vaccine design (33, 34). A liposomal formulation of lipopeptide BLP25 has progressed through Phase IIb clinical trials in over 100 patients with non-small cell lung carcinoma and was shown to be safe. Hepatitis B virus (HBV) core antigen and its peptide epitopes are candidates for therapeutic vaccines for HBV chronic infection and hepatocellular carcinoma (35, 36). The collagen IV peptide used in this study is used as a model peptide in the development of vaccine formulations (37, 38). These investigations confirm that, with repect to size, peptide content, and release characteristics, both liposomes and nanospheres are suitable delivery systems for therapeutic vaccines.

Table 2-1. Peptide Sources and Amino Acid Sequences

Peptide	Source	Sequence
BP16	Human MUC1	GVTSAPDTRPAPGSTA
BP24	Human MUC1	TAPPAHGVTSAPDTRPAPGSTAPP
BP25	Human MUC1	STAPPAHGVTSAPDTRPAPGSTAPP
BLP25	Human MUC1	STAPPAHGVTSAPDTRPAPGSTAPP-Lys(Pal)G
HBcAg$_{120-140}$	Hepatitis B Core Ag	VSFGVWIRTPPAYRPPNAPIL
HBcAg$_{126-140}$	Hepatitis B Core Ag	IRTPPAYRPPNAPIL
HBcAg$_{129-140}$	Hepatitis B Core Ag	PPAYRPPNAPIL
BP1-072	Collagen IV a2	EAIQPGCIGGPKGLPGLPGP

Table 2-2. Average Hydrophobicity Values of Peptides

Peptide	Average Hydrophobicity
BP16	-0.481
BP24	-0.600
BP25	-0.608
BLP25	not determined
HBcAg $_{120-140}$	0.143
HBcAg $_{126-140}$	-0.407
HBcAg $_{129-140}$	-0.450
BP1-072	-0.020

Table 2-3. Elution Conditions

Peptide	Gradient (%A)	Time (min)
BP16	100% to 60%	19
BP24	96% to 54%	18
BP25	96% to 54%	18
BLP25	75% to 0%	16
$HBcAg_{120-140}$	91% to 50%	18
$HBcAg_{126-140}$	91% to 60%	18
$HBcAg_{129-140}$	91% to 60%	18
BP1-072	83% to 55%	18

Table 2-4. Particle Size of Liposomes and PLGA Nanospheres (NS)

Peptide	Formulation	Particle Size
BLP25	NS (6,000 MW)	290 +/- 56 nm
$HBcAg_{129-140}$	Liposomes	782 +/- 94 nm
$HBcAg_{129-140}$	NS (6,000 MW)	510 +/- 147 nm
$HBcAg_{129-140}$	NS (50,000 MW)	569 +/- 238 nm

Table 2-5. Peptide Recovery from Liposomes (mean +/- SD)

Peptide	25% Ethanol	98% Ethanol	Methanol
BP16	70 +/- 2%	20 +/- 1%	45 +/- 3%
BP24	78 +/- 7%	40 +/- 1%	58 +/- 4%
BP25	82 +/- 11%	55 +/- 6%	77 +/- 4%
BLP25	10 +/- 8%	64 +/- 10%	60 +/- 13%
$HBcAg_{120-140}$	69 +/- 5%	87 +/- 6%	68 +/- 7%
$HBcAg_{126-140}$	73 +/- 4%	78+/- 6%	72 +/- 8%
$HBcAg_{129-140}$	98 +/- 9%	82 +/- 9%	73 +/- 7%
BP1-072	113 +/- 10%	82 +/- 10%	87 +/- 8%

Table 2-6. Peptide Recovery from PLGA Nanospheres (NS)

Peptide	Formulation	THF Extraction	Acetonitrile Extraction
BLP25	NS (6,000 MW)	65%	87%
$HBcAg_{129-140}$	NS (6,000 MW)	47%	93%
$HBcAg_{129-140}$	NS (50,000 MW)	45%	91%

Table 2-7. Encapsulation Efficiency of Peptide in Liposomes (mean +/- SD)

Peptide	Encapsulation Efficiency	Amount of Peptide Accounted For
BP16	13 +/- 1%	61%
BP24	13 +/- 8%	87%
BP25	8 +/- 5%	71%
BLP25	99 +/- 11%	99%
HBcAg$_{120-140}$	19 +/- 1%	71%
HBcAg$_{126-140}$	29 +/- 4%	99%
HBcAg$_{129-140}$	21 +/- 3%	82%
BP1-072	3 +/- 1%	81%

Table 2-8. Encapsulation Efficiency of Peptides in PLGA Nanospheres

Peptide	Formulation	Acetonitrile Extraction
BLP25	NS (6,000 MW)	90%
$HBcAg_{129-140}$	NS (6,000 MW)	13%
$HBcAg_{129-140}$	NS (50,000 MW)	11%

Table 2-9. Release of Peptides from Liposomes and PLGA Nanospheres

Peptide	Formulation	Extraction Method	% Peptide Retained
BLP25	NS (6,000 MW)	Acetonitrile	103%
HBcAg$_{129\text{-}140}$	Liposomes	25% Ethanol	92%
HBcAg$_{129\text{-}140}$	NS (6,000 MW)	Acetonitrile	91%
HBcAg$_{129\text{-}140}$	NS (50,000 MW)	Acetonitrile	90%

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Chapter Three

Use of a Liposome Antigen Delivery System to Alter Immune Responses *In vivo*

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3.1 Introduction

Hepatitis B core antigen (HBcAg) is an important candidate for a therapeutic vaccine as treatment for chronic hepatitis B. A peptide containing residues 129-140 of this protein has been shown to contain a T helper (Th) epitope. Following immunization of H-2^b mice with peptide emulsified with complete Freund's adjuvant (CFA), mice developed a Th2-type response as evidenced by the cytokine profiles of the T cells (1). In addition to the genetic background of the host and other factors, the method of antigen delivery greatly affects the immune response generated against the antigen. In this series of investigations, the effect of altering the formulation of a Th2-inducing antigen, HBcAg$_{129\text{-}140}$, was examined.

3.2 Materials and Methods

3.2.1 Materials

The lipids used were dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylglycerol (DMPG) (Genzyme Pharmaceuticals, Cambridge, MA, USA) and cholesterol (CHOL) (Sigma Chemical Co., St. Louis, MO, USA). The test peptides, HBcAg$_{126\text{-}140}$ and HBcAg$_{129\text{-}140}$, and the recall antigens, HBcAg$_{120\text{-}140}$ and HBcAg$_{110\text{-}140}$, were synthesized using either a Wang (2) or Rink amide methylbenhydrylamine (MBHA) resin (3) using the Fmoc/t-Butyl strategy (4). Biomira Inc. (Edmonton, Alberta, Canada) supplied all other recall antigens. The adjuvants used were monophosphoryl lipid A (MPLA) (Ribi Immunochem Research Inc., Hamilton, MT, USA), alum (Alhydrogel, Cedar Lane, Hornby, Ontario, Canada), and CFA (Sigma Chemical Co, St. Louis, MO, USA). Biomira Inc. supplied the cytokine reagents: first antibodies to IL-4 and IFN-γ, biotinylated second antibodies, and standards for each cytokine.

3.2.2 Preparation of Liposomes

Liposomes were prepared using a modified freeze-thaw method. Briefly, lipids were used at a molar ratio of 3 DPPC: 1 CHOL: 0.25 DMPG. Lipids in solution were coated onto round-bottom flasks using a rotary evaporator

(Buchi RE111 Rotavapor, Buchi Laboratiums-Technik, Switzerland). The volumes used were 1.95 ml of DPPC (16 mg/ml) in $CHCl_3$, 1.8 ml of CHOL (3.08 mg/ml) in $CHCl_3$, 2.2 ml of DMPG (1.1 mg/ml) in a mixture of methanol and $CHCl_3$ (1:3), and 200 μl of a 2 mg/ml solution of MPLA in methanol and chloroform (1:4). The flasks were incubated in a 43°C vacuum oven overnight to remove residual solvent. Lipids were rehydrated with 2 ml of PBS containing 300 μg of the immunizing antigen. Flasks were incubated in a 53°C water bath and then vortexed until lipids were removed from the sides of the flasks and the solution appeared homogenous. Five cycles of the following were performed: freeze flasks in dry ice/acetone, thaw for 40 minutes at room temperature, incubate at 41°C in water bath for 5 min, vortex for 30 s. Liposomes were collected via ultracentrifugation (150,000 x *g* for 20 minutes) (Model LB-55, Beckman Instruments Inc., Mississauga, ON, Canada), and washed twice with PBS. Supernatants (S1 and S2) were collected after each centrifugation for analysis of peptide content. The liposome pellet was resuspended in a replacement volume of PBS.

3.2.3 Analysis of Encapsulation Efficiency

Encapsulation efficiency was determined using the method described in chapter 2. Liposome pellets were dissolved in 25% ethanol in water and mixed with the internal standard phenol. The supernatant was collected for analysis by HPLC. All samples were injected into a Waters 625LC HPLC (Waters, Mississauga, ON, Canada) by hand. The HPLC was equipped with a C_{18} reverse phase column (8 x 100 mm) and an UV detector (Waters 486) set at 210 nm. The mobile phases employed were A [10% acetonitrile (Fisher Scientific) and 0.1% trifluoroacetic acid (Sigma, St. Louis, MO, USA) in water] and B (70% acetonitrile and 0.085% trifluoroacetic acid in water). Peptides were eluted using a gradient of 91-50% A over 18 minutes. Quantification of $HBcAg_{126-140}$ and $HBcAg_{129-140}$ was done using a calibration curve based on peak area. The quantity of unencapsulated peptide in the supernatants collected during liposome preparation was also determined by HPLC analysis as above.

3.2.4 Liposome Size Determination

Liposome size was determined by dynamic light scattering (model BI-90 Particle Sizer, Brookhaven Instruments Corp., Holtsville, NY, USA). The sample was diluted 100 X with buffer and size was measured at 25°C.

3.2.5 Immunization

All animal experiments were performed in compliance with CCAC regulations. Female C57BL/6J (H-2^b) mice (Jackson Laboratories, Bar Harbor, ME, USA), 8-12 weeks of age, were used for the immune response studies. In the first experiment, mice were injected subcutaneously with either 100 μl of liposomes (dose/mouse: DPPC- 2.2 mg, CHOL- 0.38 mg, DMPG- 0.17 mg, MPLA- 20 μg, $\text{HBcAg}_{126\text{-}140}$- 7.12 μg) or 100 μl alum plus 5 μg/5 μl $\text{HBcAg}_{126\text{-}140}$. In the second experiment, mice were immunized with 100 μl of liposomes (dose/mouse: DPPC- 2.2 mg, CHOL- 0.38 mg, DMPG- 0.17 mg, MPLA- 20 μg, $\text{HBcAg}_{129\text{-}140}$- 6.78 μg), 100 μl alum plus 100 μl of a 1 mg/ml solution of $\text{HBcAg}_{129\text{-}140}$, or 100 μl of CFA emulsified with 100 μl of a 1 mg/ml solution of $\text{HBcAg}_{129\text{-}140}$. Mice in the second experiment received a booster injection one week after the initial immunization.

3.2.6 Immunological Assays

3.2.6.1 T Cell Proliferation

Ten days after the final immunization, mice were sacrificed and the inguinal lymph nodes were removed. Lymph node cells were passed through nylon wool (Robbins Scientific, Sunnyvale, CA, USA) columns to enrich for T cells (6). Spleen cells from naïve mice were used as antigen presenting cells (APC) after being irradiated with 3000 rads in a ^{137}Cs irradiator. In a 96 well flat bottom plate, 5 X 10^5 T cells per well were incubated at 37°C with 1 X 10^6 APC per well along with relevant or irrelevant peptides as the recall antigens or 3 μg/ml concanavalin A (Sigma Chemical Co.) as a positive control. After 72 hours, the cells were pulsed with 1 μCi ^{3}H-thymidine 50 μl of media per well (Amersham Canada, Oakville, Ontario, Canada) and 24 hours later the

cells were harvested. In the first experiment, cells were harvested onto a Skatron 1731 filtermat (Skatron Micro96 Harvester, Sterling, VA, USA) and the radioactivity incorporated into the cells was measured using a Wallac 1410 Liquid Scintillation Counter (Wallac, Turku, Finland). In the second experiment, the cells were harvested onto a filter mat using a Mach III Harvester 96 and the filter was read in a Microbeta Trilux reader (Wallac). The proliferation of different groups was compared using their stimulation indexes (SI). SI is equal to the cpm of T cells incubated with APC and antigen/the cpm of T cells incubated with APC without antigen.

3.2.6.2 Cytokine Analysis

In a 96 well flat bottom plate, T lymphocytes (collected from primed mice in the T cell proliferation experiment described above) were incubated at 37°C with APC and either relevant or irrelevant recall antigens. Supernatants were collected from the wells after 24, 48, and 72 hours of incubation and frozen at -20°C for later analysis. Supernatants were analyzed for levels of IFN-γ and IL-4 by sandwich ELISA performed in 96 well microtiter plates (NUNC Maxisorp Immunoplates, Gibco-BRL, Burlington, Ontario, Canada). The first antibody to IFN-γ (R46.A2) was diluted to 1.5 μg/ml with PBS and 50 μl was added to each well. The first antibody to IL-4 (11B11) was diluted to 0.5 μg/ml and 50 μl was added to each well. Plates were incubated for 30 min at 37°C and washed once with TPBS [PBS containing 0.05% v/v Tween 20 (Sigma)]. Undiluted supernatant samples were added at 50 μl/well. Recombinant IFN-γ was serially diluted with media to concentrations from 5000 to 156 pg/ml and 50 μl was added to each well. Recombinant IL-4 was diluted to 1666 to 52 pg/ml and 50 μl was added to each well. Plates were incubated for 45 min at 37°C and washed twice with TPBS. Biotinylated second antibodies (IFN-γ- XMG1.2; IL-4- BVD6.24G2) were diluted to 0.05 μg/ml and 50 μl was added to each well. Plates were incubated for 45 min at 37°C and washed three times with TPBS. Peroxidase-conjugated strepavidin (Jackson Immunoresearch Lab Incorp., West Grove, PA, USA) was diluted to

200 ng/ml and 50 μl was added to each well. Plates were incubated for 30 min and washed 4 times. ABTS substrate (KPL, Gaithersburg, MD, USA) solutions A and B were mixed in equal amounts and 100 μl was added to each well. After 15 minutes of incubation at room temperature, the absorbance at 405 nm was read using a microplate reader (Molecular Devices, Menlo Park, CA, USA). The concentration of IFN-γ or IL-4 (pg/ml) in the test samples was determined by comparing the absorbance values of the test samples with that of the reference standards using a standard curve.

3.2.7 Statistical Analysis

The results of the immunological assays are expressed as the mean +/- standard deviation (S.D.) of triplicate wells. An unpaired student's t test was used to compare sample means and assess statistical significance. P values < 0.05 were considered significant.

3.3 Results

3.3.1 Characterization of Liposomes

The encapsulation efficiency of the $HBcAg_{126-140}$ in the liposomes was 28.75 +/- 3.75% (mean +/- range). The average size of the liposomes was 795 +/-25 nm (mean +/- range). The encapsulation efficiency of $HBcAg_{129-140}$ in the liposomes was 19.67 +/- 1.82% (mean +/- SD). The average size of the liposomes was 847 +/-49 nm (mean +/- range).

3.3.2 Characterization of Immune Response After Single Immunization

Antigen-specific T cell responses induced by a single subcutaneous immunization with the liposomal formulation or a control alum formulation of $HBcAg_{126-140}$ were evaluated by an *ex vivo* T cell proliferation assay (Figure 3-1). T cells from mice immunized with liposomes showed a strong *ex vivo* recall response (SI = 10-12) to the peptide used for immunization ($HBcAg_{126-}$

$_{140}$) and to a related, longer peptide ($HBcAg_{110-140}$), but not to irrelevant peptides such as BP24 or BP1-037 ($p<0.001$). T cells from the mice immunized with an alum formulation of peptide showed no antigen-specific recall response *ex vivo* ($p>0.05$).

The magnitude of the antigen-specific T cell proliferative response induced by liposomal formulation was also dependent on the concentration of the recall peptide in the range of 1 to 25 μM (Figure 3-2). A significant response was observed even at as low a level of recall antigen as 1 μM antigen/well.

The type of T cell response induced was further characterized by quantification of IFN-γ and IL-4 produced during T cell proliferation. T cells from mice immunized with the liposomal formulation showed a significantly higher production of IFN-γ in response to the immunizing peptide than to irrelevant peptides (Figure 3-3). IL-4 levels were below the limits of the assay (52 pg/ml) (data not shown). T cells from mice immunized with alum and $HBcAg_{126-140}$ did not produce any measurable IFN-γ in response to relevant peptide in comparison with irrelevant recall antigens. IL-4 levels were below the limits the assay for the alum group as well (data not shown). These results indicate that a liposomal formulation of $HBcAg_{126-140}$ induced an antigen-specific Th1-type response whereas the alum formulation failed to induce detectable antigen-specific T cell activation.

3.3.3 Characterization of Immune Response After Two Immunizations

Further investigation into the ability of liposomes to elicit a Th1-type response to a Th2-inducing peptide required a more effective control formulation. In this model, with the dose and immunization schedule employed, alum did not elicit any antigen-specific T cell proliferation. The formulation used by the investigators who identified $HBcAg_{129-140}$ as a Th2-type peptide was an emulsion of peptide and CFA (1) and all further work used this formulation. Another change made to the experimental procedure

was the use of was $HBcAg_{129-140}$ rather than $HBcAg_{126-140}$ because the 12mer is the minimal epitope for a T cell response.

In this experiment, mice received two immunizations with the formulations, one week apart. The formulation of alum with an increased dose of peptide did elicit an antigen-specific T cell response after two immunizations but it was considerably less intense of a response (SI= 2) than was elicited by either the CFA (SI=11) or the liposomal formulations (SI=22) (Figure 3-4). There was little difference in the strength of responses to the CFA and liposomal formulations of peptide. In both cases, the response was antigen-specific. The response seen to the recall antigen $HBcAg_{120-140}$ was as strong or stronger than the response to $HBcAg_{129-140}$. It is possible that its larger size offered better protection from enzymatic degradation or an advantage for antigen processing and presentation *in vitro*.

The type of T cell response was also characterized. The liposomal formulation elicited significantly more IFN-γ production than did either the alum or CFA formulations ($p<0.01$) (Figure 3-5). It was of particular interest that, although the magnitude of the T cell response to CFA was similar to that seen with the liposomal formulation, there was no comparable production of IFN-γ. Levels of IL-4 were below the limits of detection of the assay (data not shown) for all formulations.

3.4 Discussion

This set of investigations examines the effect of antigen delivery systems on the type of immune response generated against a peptide *in vivo*. In the case of viral infection, such as infection by the hepatitis B virus, the ability to manipulate the type of response by immunotherapy could be crucial for recovery from disease. One approach to the manipulation of the immune response is through the use of antigen delivery systems such as liposomes and nanospheres, which have been shown to affect the immune response generated by an antigen (7-9). If the immune response to the virus can be

changed into a Th1-type response by formulating antigen into liposomes, the clinical outcome of hepatitis B infection may be altered.

The peptide consisting of residues 129-140 of the hepatitis B core protein ($HBcAg_{129-140}$) has been shown to evoke a Th2-type response in C57BL/6 mice after its immunization in an emulsion with CFA. CFA is generally regarded as strong 'Th1-inducing adjuvant'. Development of a Th2-type response against this peptide even when administered in CFA indicates that this peptide has a strong 'Th2 bias' and justifies its description as a 'Th2-inducing peptide'. In these experiments, C57BL/6 mice were immunized with control formulations of $HBcAg_{126-140}$ or $HBcAg_{129-140}$ in alum or CFA or with peptide and MPLA encapsulated in liposomes. The results demonstrate that immunization with liposomes led to a strong Th1-type response, based on the antigen-specific production of IFN-γ. Although the formulation of CFA and $HBcAg_{129-140}$ elicited a T cell response of the same magnitude as that caused by the liposomes and despite the reputation of CFA as a Th1-inducing adjuvant, there was no IFN-γ production seen after immunization with the CFA emulsion.

These experiments demonstrate that by changing the formulation of the immunizing peptide we can elicit a Th1-type response to a Th2-inducing peptide. If we can use this formulation to overcome an existing Th2-type response in favour of a Th1-type response, we can potentially change the outcome of a hepatitis B infection from the development of chronic disease and its sequelae to recovery and clearance of the infection at the acute stage. Such antigen delivery approaches may also be used in the formulation of therapeutic vaccines for other viral infections and cancer where Th1-type responses favour recovery and Th2-type responses favour disease progression.

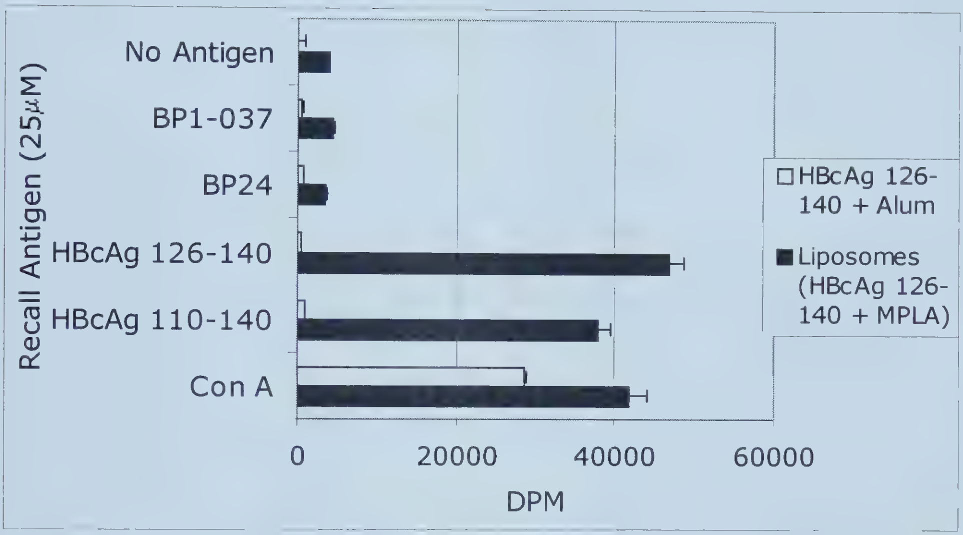

Figure 3-1. Antigen specificity of T cell proliferative responses *ex vivo*. C57BL/6 mice were immunized with $HBcAg_{126\text{-}140}$ peptide in a liposomal formulation or with alum. The antigen-specificity of the response was determined by comparing the levels of proliferation (shown as ^3H-thymidine incorporation) after incubation with relevant antigens ($HBcAg_{126\text{-}140}$ or $HBcAg_{110\text{-}140}$) to that obtained after incubation with irrelevant antigens (BP1-037 or BP24). T cell cultures incubated with Con A acted as positive controls while T cell cultures incubated with no antigen were used to measure background proliferation. The experiment was performed once.

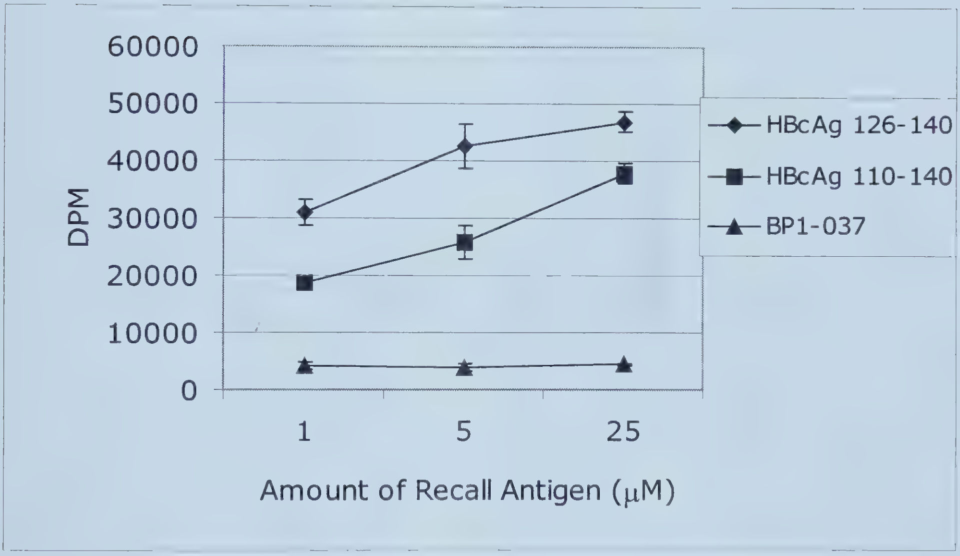

Figure 3-2. Effect of the concentration of recall antigens on antigen-specific T cell proliferation *ex vivo*. A T cell proliferation assay was performed with T cells of mice immunized with liposomes containing $HBcAg_{126\text{-}140}$. The concentration of recall antigens was varied.

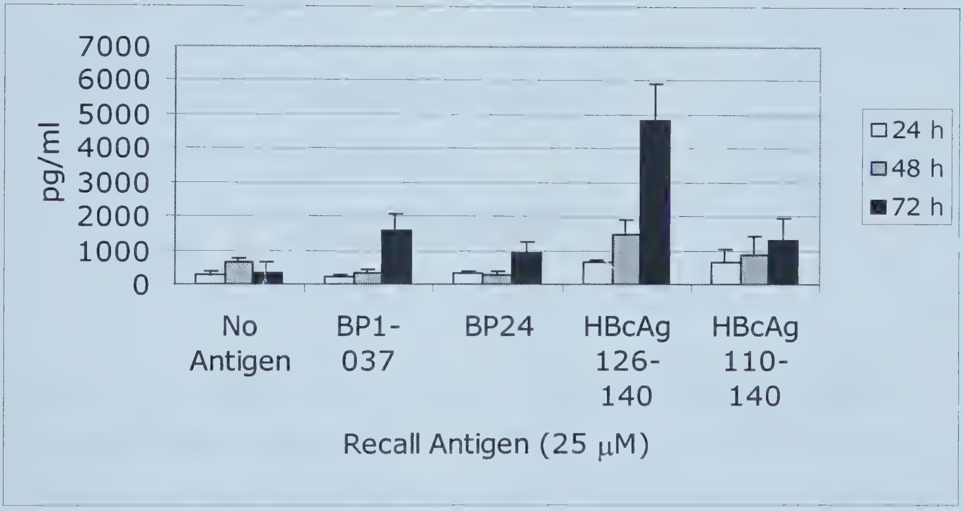

Figure 3-3. IFN-γ levels associated with T cell proliferation *ex vivo*.

The levels of IFN-γ in the supernatant of T cell cultures were determined by ELISA after 24, 48, and 72 hours of incubation with recall antigens.

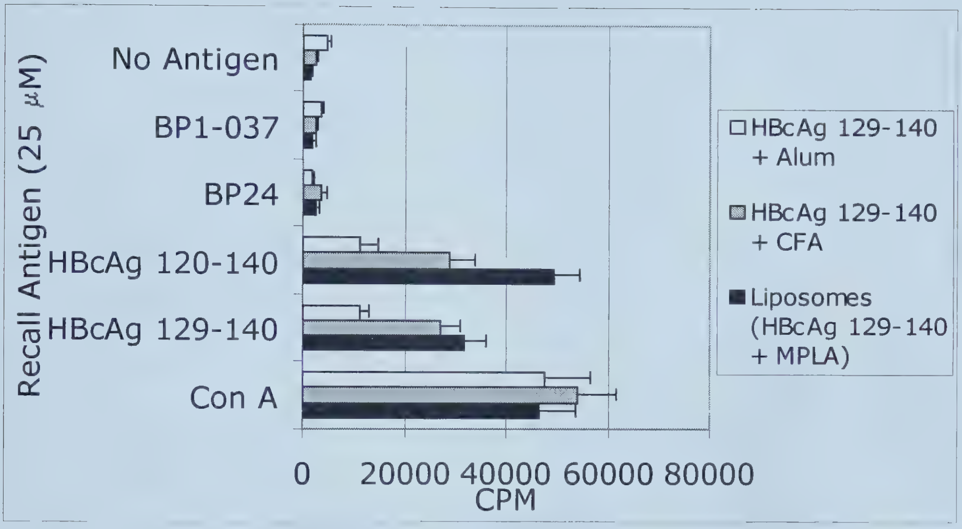

Figure 3-4. Antigen specificity of T cell proliferative responses *ex vivo* after two immunizations. C57BL/6 mice were immunized with $HBcAg_{129-140}$ peptide in liposomes, emulsified with CFA or with alum. The antigen-specificity of the response was determined by comparing the levels of proliferation (shown as ^{3}H-thymidine incorporation) after incubation with relevant antigens ($HBcAg_{129-140}$ or $HBcAg_{120-140}$) to that obtained after incubation with irrelevant antigens (BP1-037 or BP24). T cell cultures incubated with Con A acted as positive controls while T cell cultures incubated with no antigen were used to measure background proliferation. Representative results from one of three experiments are shown.

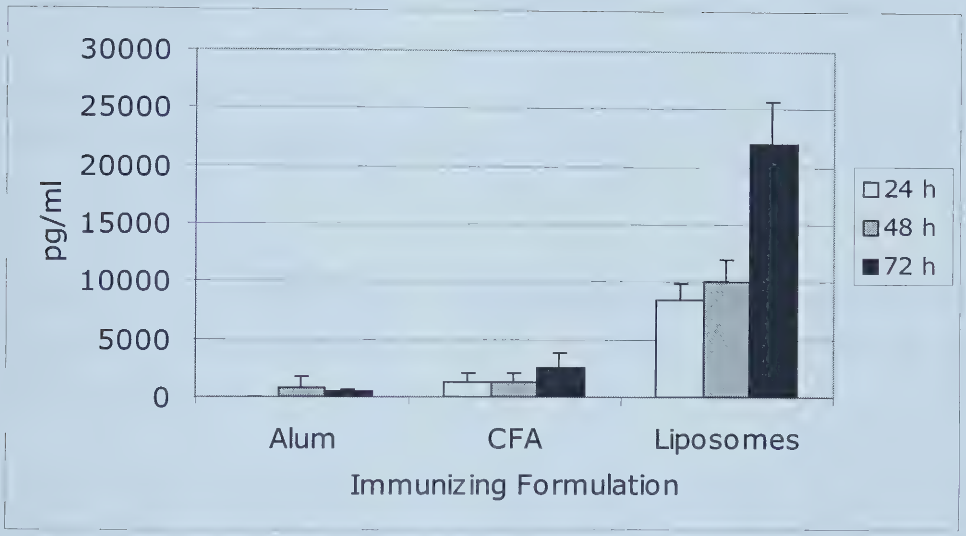

Figure 3-5. Antigen-specific T cell production of IFN-γ in response to HBcAg129-140. The levels of IFN-γ in the supernatant of T cell cultures in presence of recall antigens for 24, 48, or 72 hours were determined by ELISA. Representative results from one of three experiments are shown.

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Chapter Four

Biodegradable Nanosphere Delivery of a Th2 Peptide for Induction of Th1 Immune Responses

4.1 Introduction

Hepatitis B is a disease in which the type of immune response developed by the patient is crucial in determining disease outcome. If the immune response in patients with chronic hepatitis B could be switched from a Th2-type to a Th1-type, the clinical outcome of the disease may be altered. Antigen formulated in a particulate delivery system, such as PLGA nanospheres, can be used as a therapeutic vaccine that could accomplish this switch in the type of immune response and lead to disease resolution.

A peptide consisting of residues 129-140 of the hepatitis B core antigen (HBcAg) has been shown to generate a Th2-type response, based on cytokine profile and antibody isotype, in mice of the H-2^b haplotype after immunization with a formulation of peptide emulsified with complete Freund's adjuvant (CFA) (1). $HBcAg_{129-140}$ is a therapeutically relevant antigen that allows the study of how formulation of the peptide can be used to alter the response to the antigen.

In this series of investigations, the ability of antigen encapsulated in PLGA nanospheres to alter the response elicited against the antigen was examined. In addition, the capability of a PLGA nanosphere formulation to elicit a Th1-type response after the immune system had been imprinted to give a Th2-type response was explored.

4.2 Materials and Methods

4.2.1 Preparation of PLGA Nanospheres

PLGA nanospheres were prepared using a water/oil/water solvent evaporation technique (2). Briefly, 100 μl of a 2 mg/ml solution of $HBcAg_{129-140}$ (synthesized and provided by Dr. D. S. Wishart's laboratory, University of Alberta, Edmonton, AB, Canada) was emulsified in 300 μl of chloroform (Fisher Scientific, Nepean, ON, Canada) containing 100 mg of PLGA (BPI, Birmingham, AL, USA) polymer (lactic acid: glycolic acid ratio 50:50;

MW=6000 or 50,000) and 200 μl of a 2 mg/ml solution of monophosphoryl lipid A (MPLA) (Biomira Inc, Edmonton, AB, Canada) using a micro tip sonicator (Model XL2010, Heat Systems INC., Farmingdale, NY, USA). The resulting primary emulsion was added into 2 ml of 9% w/v polyvinyl alcohol (PVA) (87-89% hydrolyzed, MW 31-50,000; Aldrich Chemical Co. Inc., Milwaukee, WI, USA) and was further sonicated to form the secondary emulsion. This emulsion was added drop wise to 8 ml of 9% w/v PVA and stirred for 3 h to evaporate the chloroform. The nanospheres were then collected by ultracentrifugation (40,000 x *g*, 10 minutes at 20°C), washed twice with distilled water (20 ml) to remove residual PVA, resuspended in 5 ml distilled water, and freeze-dried for 3 days. Nanospheres were stored at −20°C until needed.

4.2.2 Nanosphere Size Determination

Nanosphere size was determined by dynamic light scattering (Zetasizer 3000 HSa, Malvern Instruments Ltd., San Bernadino, CA, USA). Five milligrams of nanospheres were suspended in 1 ml of PBS. Size was measured at 25°C.

4.2.3 Analysis of Encapsulation Efficiency

Ten milligrams of nanospheres were dissolved in 1 ml of acetonitrile (Caledon Laboratories Ltd., Georgetown, ON, Canada) and vortexed for 10 minutes. The sample was centrifuged for 10 minutes at 10,300 x *g*. The supernatant was discarded and the pellet was dissolved in 500 μl distilled water. The peptide concentration in the sample was determined by comparing the UV absorbance of the peptide with a standard curve (R^2 > 0.998) generated under the same conditions. Two hundred milliliters of the sample was injected into a Waters 625LC HPLC (Waters, Mississauga, ON, Canada) using a Gilson 234 Autoinjector (Mandel Scientific Co., Guelph, ON, Canada). The HPLC was equipped with a C_{18} reverse phase column (8 x 100 mm) and an UV detector (Waters 486) set at 210 nm. The mobile phases employed were A [10% acetonitrile (Fisher Scientific) and 0.1% trifluoroacetic acid (Sigma, St. Louis, MO, USA) in water] and B (70% acetonitrile and 0.085%

trifluoroacetic acid in water). The peptide was eluted using a gradient of 9 – 50% B over 18 minutes. EE was calculated using the weight of the nanospheres and the amount of peptide incorporated.

4.2.4 Immunization of Mice

All animal experiments were performed in accordance with CCAC regulations. Female C57BL/6 (H-2^b) mice (Charles River Laboratories, Quebec, Canada) aged 8 to 12 weeks were used for the immune response studies. For the investigations of immune responses in naïve mice, there were two groups of 6 mice each: i) test mice were immunized on day 0 with 5 mg nanospheres containing 3.5 μg of $HBcAg_{129\text{-}140}$ and 20 μg of MPLA in 200 μl PBS and two weeks later received a booster immunization with the same formulation ii) control mice were immunized on day 0 with an emulsion of 100 μl CFA (Sigma) and 100 μl of $HBcAg_{129\text{-}140}$ (2 mg/ml) solution and received a booster injection two weeks later of 200 μl incomplete Freund's adjuvant (IFA) (Sigma) and peptide. In the first experiment, nanospheres were prepared with PLGA with a molecular weight of 50,000, while in the second experiment, nanospheres were prepared with PLGA with a molecular weight of 6000. For the investigation of immune responses in mice whose immune systems were imprinted for a Th2-type response, on day 0, twelve mice were immunized with an emulsion of 100 μl CFA + 100 μl $HBcAg_{129\text{-}140}$. Two weeks later, six control mice were immunized with an emulsion of IFA and peptide while six test mice were immunized with 5 mg nanospheres containing 3.5 μg $HBcAg_{129\text{-}140}$ and 20 μg MPLA in PBS. All immunizations were via subcutaneous injection in the flank.

4.2.5 Immune Response Studies

4.2.5.1 T Cell Proliferation

The magnitude and specificity of the immune responses elicited by immunization were determined using a T cell proliferation assay. In this assay, the recall responses of T cells isolated from immunized mice were measured *in vitro* against relevant and irrelevant peptide antigens.

Ten days after the second immunization, mice were sacrificed by CO_2 asphyxiation and cervical dislocation. The inguinal lymph nodes were removed, the lymph node cells isolated and placed in RPMI-10 [RPMI 1640 medium (Gibco BRL, Burlington, ON, Canada) supplemented with 1 mL L-glutamine (Gibco BRL), 50 units of penicillin (Gibco BRL), 50 μg/ml of streptomycin (Gibco BRL), 75 μg/ml of gentamicin (Gibco BRL), 10 mM HEPES (Gibco BRL), and 10% fetal bovine serum (Gibco BRL)]. Lymph node cells were passed through nylon wool (type 200 L, Robbins Scientific, Sunnyvale, CA, USA) columns to enrich for T cells (3). The T cells were eluted off of the columns using warm RPMI-10. The purified T cell were then centrifuged at 200 x *g* for 7 minutes and resuspended at 1 x 10^7 cells/ml in RPMI-10.

In 96 well, flat-bottom, micro titer plates (Costar, Cambridge, MA, USA), the T cells were plated at a concentration of 3 x 10^5 T cells/well. Recall antigens were added to the wells at a concentration of 25 μM. Antigen presenting cells (APC) were added to the wells at a concentration of 1 x 10^6 cells/well. The recall antigens included one irrelevant peptide and two relevant peptides: BP24 (Biomira Inc.) with the sequence TAPPAHGVTSAPDTRPAPGSTAPP; the immunizing antigen, $HBcAg_{129-140}$, with the sequence PPAYRPPNAPIL; and $HBcAg_{126-140}$ with the sequence IRTPPAYRPPNAPIL (core peptides were prepared by Dr. David S. Wishart's laboratory, University of Alberta, Edmonton, AB, Canada). In addition to the wells containing recall antigens, there were also wells used to measure background proliferation containing T cells and APC without any antigen and positive control wells set up with T cells, APC, and 2 μg/well concanavalin A (Con A; Sigma) in place of a recall antigen.

APC were obtained from the spleens of unimmunized syngeneic mice. Spleens were removed and the cells collected in RPMI-10. Spleen cells were irradiated with 3000 rad in a ^{137}Cs irradiator. Irradiated spleen cells were

washed thoroughly with RMPI-10 media, collected by centrifugation at 200 x *g* for 7 minutes, and resuspended in RPMI-10 at a concentration of 1 x 10^7 cells/ml before being added to the 96 well plates.

After 72 hours of incubation at 37°C/5% CO_2, 1 μCi ^3H-thymidine (Amersham Canada, Oakville, Ontario, Canada) in 50 μl RPMI-10 was added to each well. After 24 hours of incubation, the cells were harvested onto a filter mat using a Mach III Harvester 96 and the filter was read in a Microbeta Trilux reader (Wallac, Turku, Finland). T cell proliferation was measured as the amount of incorporated radioactivity in counts per minute (cpm). The proliferation of different groups was compared using their stimulation indexes (SI). SI is equal to the cpm of T cells incubated with APC and antigen/the cpm of T cells incubated with APC without antigen.

4.2.5.2 Cytokine Analysis

Culture setup was the same as for the T cell proliferation. Supernatants were collected from the wells after 24, 48, and 72 hours of incubation and frozen at −20°C until they were analyzed. Levels of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) in the supernatants were determined by sandwich ELISA. Briefly, 96 well micro titer plates (NUNC Maxisorp Immunoplates, Gibco-BRL, Burlington, Ontario, Canada) were coated with 50 μl/well of a primary antibody: the primary antibody against IFN-γ (R46.A2; Biomira Inc.) was diluted to 1.5 μg/ml with PBS; the primary antibody against IL-4 (11B11; Biomira Inc.) was diluted to 0.5 μg/ml. Plates were incubated for 30 min at 37°C and washed once with TPBS [PBS containing 0.05% v/v Tween 20 (Sigma)]. A set of standards was prepared for each cytokine. Recombinant IFN-γ (Biomira Inc.) was serially diluted in RPMI-10 to concentrations from 5000 to 156 pg/ml. Recombinant IL-4 (Biomira Inc.) was diluted with RPMI-10 to 1000 to 25 pg/ml. Each plate received duplicate wells of the appropriate set of standards. Standards were added at 50 μl/well. Negative control wells received 50 μl of RMPI-10 media. In the first assay, undiluted supernatants were added at 50 μl/well. Some of the

supernatants had to be reanalyzed after being diluted (10-50 fold) with RPMI-10 because the readings were above the upper limit of detection of the plate reader. Plates were incubated for 45 min at 37°C and were washed twice with TPBS. Biotinylated secondary antibodies were added at 50 μl/well: the secondary antibody against IFN-γ (XMG1.2, Biomira Inc.) was diluted to 0.05 μg/ml with a solution of 1% bovine serum albumin (BSA) (Sigma) in TPBS; the secondary antibody against IL-4 (BVD6.24G2, Biomira Inc.) was diluted to 0.2 μg/ml. Plates were incubated for 45 min at 37°C and then washed three times with TPBS. Peroxidase-conjugated streptavidin (Jackson Immunoresearch lab Inc., West Grove, PA, USA) was diluted to 200 ng/ml in 1% BSA in TPBS and 50 μl was added to each well. Plates were incubated for 30 min and then washed 4 times with TPBS. Peroxidase solution (KPL, Gaithersburg, MD, USA) was combined in equal parts with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (KPL) and 100 μl of the mixture was added to each well. After 2 minutes, an optical density (OD) reading was taken at 650 nm using a microplate reader (Powerwave 340, Bio-Tek Instruments Inc., Winoski, VT, USA). Once the OD reading reached 0.8, 100 μl of 1 M phosphoric acid (BDH Inc., Toronto, ON, Canada) was added to each well and an OD reading was performed at 450 nm.

4.2.6 Statistical Analysis

The results are expressed as the mean +/- standard deviation of triplicate wells. An unpaired student's t test was used to compare sample means and assess statistical significance. P values < 0.05 were considered significant.

4.3 Results and Discussion

4.3.1 Characterization of HBcAg$_{129-140}$ Nanospheres

Nanospheres containing HBcAg$_{129-140}$ and MPLA measured 510 +/- 147 nm in diameter. Based on the HPLC results, the encapsulation efficiency was 17.5% and peptide loading was 0.07% w/w of HBcAg$_{129-140}$ entrapped per dry weight of nanospheres. Greater than 90% of peptide was retained in the nanospheres after 24 hours of incubation of the nanospheres in PBS at 37°C.

4.3.2 Characterization of Immune Response after Immunization of Naïve Mice with Nanospheres containing $\text{HBcAg}_{129\text{-}140}$ and MPLA

After immunization with the nanosphere and CFA formulations, the antigen-specific T cell proliferation *in vitro* was measured. In this first experiment, the nanosphere formulation consisted of PLGA with a molecular weight of 50,000. T cells from mice immunized with the CFA/peptide emulsion proliferated in an antigen-specific manner. T cells from mice immunized with PLGA nanospheres containing $\text{HBcAg}_{129\text{-}140}$ and MPLA did not demonstrate any significant antigen-specific proliferation (Figure 4-1). This lack of response may be due to a lack of antigen release from the nanospheres in the APC *in vivo*. Because of the lack of a proliferative response to the high molecular weight PLGA nanospheres, further studies were carried out using PLGA with a molecular weight of 6000.

After immunization with the low MW nanosphere formulation of $\text{HBcAg}_{129\text{-}140}$ and the peptide/CFA emulsion, the antigen-specific T cell proliferation was measured. T cells from both groups of mice showed significant antigen-specific proliferation (Figure 4-2). There was no significant difference in the amount of proliferation between the mice that were immunized with nanospheres and the mice that received the CFA formulation.

In order to characterize the type of immune response, supernatants from cell cultures were collected and analyzed for IFN-γ and IL-4 content by ELISA. IL-4 results were in all cases below the limits of detection of the assay (data not shown). T cells from mice immunized with nanospheres containing $\text{HBcAg}_{129\text{-}140}$ and MPLA produced significantly greater amounts of IFN-γ than T cells from mice immunized with CFA and peptide (Figure 4-3). This difference was seen at all time points ($p< 0.0001$).

As reported in the literature, immunization with the control formulation of a CFA/$\text{HBcAg}_{129\text{-}140}$ emulsion did not generate a Th1-type response even though CFA is considered to predispose the immune system towards

developing a Th1-type response. By formulating a therapeutic peptide known to give a Th2-type response in H-2^b mice into nanospheres along with the adjuvant MPLA, it was demonstrated that it is possible to elicit a Th1-type response to the peptide in C57BL/6 mice. This is an important finding since a Th1-type response against the hepatitis B virus is necessary for recovery from infection.

4.3.3 Characterization of the Immune Response in Mice Imprinted for a Th2-type Response After Immunization With Nanospheres containing HBcAg$_{129-140}$ and MPLA

The results described in the previous section are useful but not entirely therapeutically relevant. Although it is helpful to demonstrate that the formulation of a peptide can alter the type of immune response generated by immunization with the peptide, the experiment discussed was performed in naïve mice in the absence of an ongoing immune response. This lack of an ongoing immune response is in direct contrast to the therapeutic situation where a patient has an ongoing Th2-type response against the virus. An effective therapeutic vaccine against the hepatitis B virus must be able to switch an ongoing Th2-type response to a Th1-type response. In order to examine the potential of a PLGA nanosphere formulation of HBcAg$_{129-140}$ to alter the type of immune response after the response has been established, mice were first immunized with the control formulation of CFA + HBcAg$_{129-140}$ in order to imprint their immune systems with a non-Th1-type immune response. Two weeks later, mice in the test group were immunized with nanospheres containing HBcAg$_{129-140}$ and MPLA while mice in the control group were immunized with an IFA/HBcAg$_{129-140}$ emulsion. The immune responses in the mice were measured and characterized.

The antigen-specific T cell proliferative response to recall antigens *in vitro* was measured as in the above experiment. T cells from mice that received the nanosphere formulation as the second injection and T cells from mice that received the IFA/HBcAg$_{129-140}$ emulsion as the second injection

proliferated significantly in response to the relevant recall antigens (Figure 4-4). There was no significant difference between the groups with respect to the amount of T cell proliferation or the extent of the immune response.

The production of IFN-γ and IL-4 by the T cells *in vitro* was again examined in order to characterize the type of immune response generated. Levels of IL-4 were below the limits of detection of the assay (data not shown). Despite the similar levels of T cell response seen in the two groups of mice, T cells from mice that received the nanosphere formulation after being imprinted for a Th2-type response produced significantly more antigen-specific IFN-γ than mice who received a booster immunization with the IFA/HBcAg$_{129-140}$ emulsion (Figure 4-5) ($p< 0.0001$). Both groups of mice established a strong T cell response to the hepatitis B core peptide but only mice immunized with the nanosphere formulation established the Th1-type response that is necessary for recovery from hepatitis B chronic infection. The ability of the nanosphere formulation to alter the type of immune response generated against a peptide in an immune system that had been already primed for a Th2-type response is a significant finding for the development of therapeutic vaccines.

The change that was made to the conventional immunization protocol gives rise to a more therapeutically relevant experimental design. Therapeutic vaccines are not administered to healthy, naïve patients; they are administered to patients who are combating an infection and who have an ongoing immune response against the pathogen. Whether the aim of the therapeutic vaccine is to alter the type of response or to alter the epitopes that the patients' immune systems focus on, it is crucial to study the T cell response in an environment as similar to the therapeutic situation as possible. For diseases such as hepatitis B, where an animal model is not readily available and the immune response in animals does not necessarily parallel the situation seen in humans with chronic infection, establishing an ongoing Th2-type immune response in mice before testing therapeutic vaccine formulations allows the immune response generated by the vaccine

to be characterized and the potential effectiveness of the vaccine to be evaluated in a situation closer to the therapeutic situation than the traditional immunization protocol for T cell proliferation.

In this set of investigations, the ability of a nanosphere formulation of a peptide from the hepatitis B core antigen, identified in the literature as eliciting a Th2-type response when mice of the H-2^b haplotype are immunized with a CFA formulation (1), to alter the immune response to the peptide was examined. It was demonstrated that, compared to immunization with a CFA/$HBcAg_{129-140}$ emulsion, immunization with a formulation of $HBcAg_{129-140}$ in PLGA nanospheres did not change the extent of the T cell response but did change the type of response to a strong Th1-type response. A further study was performed in order to examine whether the nanosphere formulation of the peptide could alter an ongoing immune response, in an attempt to mimic the situation of a chronic hepatitis B patient with an established Th2-type response. It was found that mice that were imprinted for a Th2-type response through immunization with a CFA/$HBcAg_{129-140}$ emulsion could generate a strong Th1-type response after immunization with nanospheres containing $HBcAg_{129-140}$ and MPLA. In contrast to earlier experiments with this peptide, the presence of IL-4 was not demonstrated in our assay system; however, the relative lack of IFN-γ production in the control group combined with the dramatic increase of IFN-γ production achieved in both naïve mice and mice primed to give a Th2-type response, allows us to state that the nanosphere formulation can elicit a Th1-type response, even in the presence of an ongoing non-Th1-type response.

There are several possible mechanisms for this ability of the nanosphere formulation to elicit a Th1-type response. It has been shown that DC transfected with IL-12 can switch a Th2-type response to a Th1-type response (4). MPLA is capable of causing the production of IL-12 by DC (5). The co-delivery of the peptide antigen and the adjuvant to the same DC enhances the effect on the immune system; it allows the peptide antigen to

be presented to the T cells by an antigen-presenting cell that is producing IL-12 and that is capable of stimulating a Th1-type response. Soluble forms of antigen delivery cannot achieve this co-delivery.

A Th1-type response against the hepatitis B virus leads to disease resolution (6). Patients who develop a Th2-type response against the virus develop chronic infection, often leading to cirrhosis, hepatocellular carcinoma, and death (6). In order to be an effective treatment for chronic hepatitis B, a therapeutic vaccine against HBV must switch the immune response in a patient towards a Th1-type response in the presence of an ongoing immune response. The ability of PLGA nanospheres to alter the type of immune response elicited by a peptide, even in the context of an ongoing immune response, makes PLGA nanospheres a strong candidate for the formulation of therapeutic vaccines.

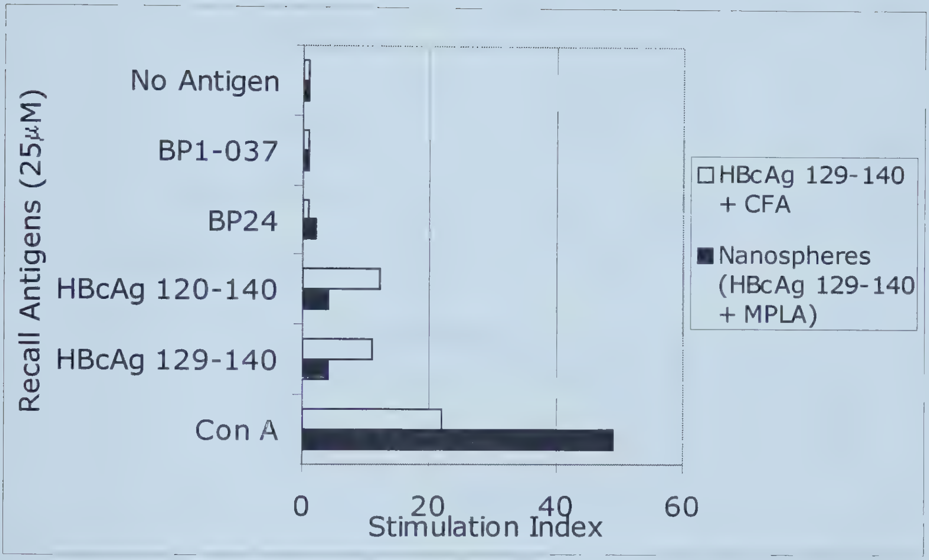

Figure 4-1. T cell proliferation after immunization of naïve mice with high molecular weight PLGA nanospheres. Naïve mice were immunized with HBcAg$_{129\text{-}140}$ either emulsified with CFA or encapsulated along with MPLA in PLGA (MW=50,000) nanospheres. The proliferative response of T cells from immunized mice to a panel of recall antigens was measured by ^{3}H-thymidine incorporation. Stimulation Index (SI) = cpm of test group/background cpm of wells without antigen. Representative results from one of two experiments are shown.

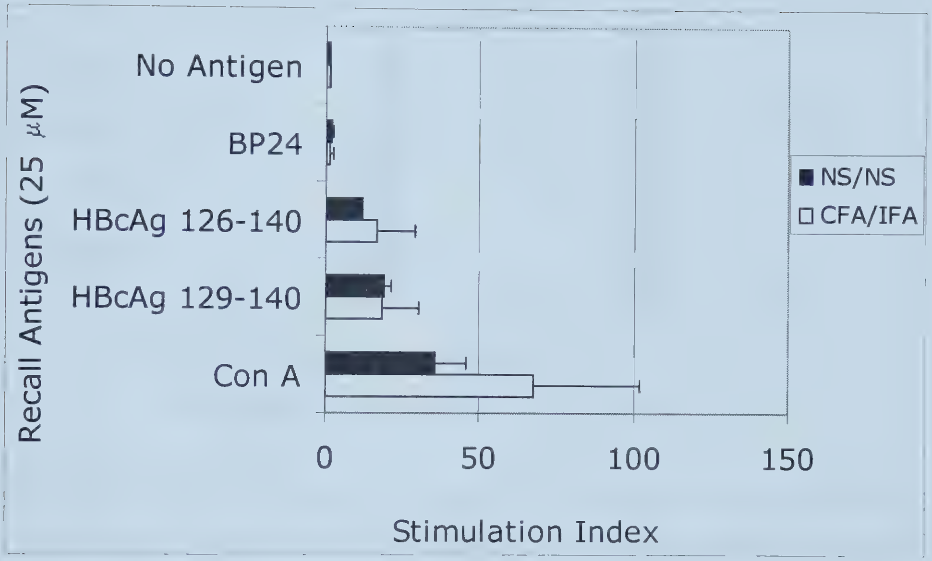

Figure 4-2. T cell proliferation after immunization of naïve mice. Naïve mice were immunized with HBcAg$_{129\text{-}140}$ either emulsified with CFA or encapsulated along with MPLA in PLGA (MW=6000) nanospheres. The proliferative response of T cells from immunized mice to a panel of recall antigens was measured by ^{3}H-thymidine incorporation. Stimulation Index (SI) = cpm of test group/background cpm of wells without antigen. The background cpm for the CFA group was 383 +/- 120. The background cpm for the nanosphere group was 2819 +/- 816. Representative results from one of two experiments are shown.

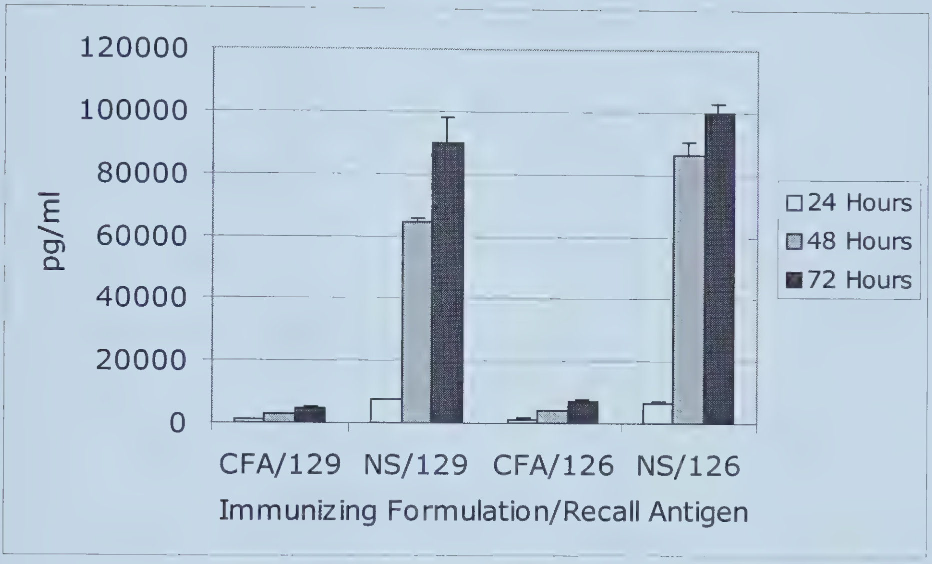

Figure 4-3. Interferon-γ production after immunization of naïve mice. Mice were immunized with $HBcAg_{129-140}$ either emulsified with CFA or encapsulated among with MPLA in PLGA (MW=6000) nanospheres. The production of IFN-γ by T cells from immunized mice in response to recall antigen was measured by ELISA. Values in pg/ml were calculated using a standard curve of known quantities. Immunizing formulation: CFA=complete Freund's adjuvant and peptide, NS=nanosphere formulation of peptide. Recall antigen: 129= $HBcAg_{129-140}$, 126= $HBcAg_{126-140}$. The background production of IFN-γ for the CFA group was 678 +/- 145 pg/ml (24 h), 279 +/- 27 pg/ml (48h), and 1193 +/- 136 pg/ml (72 h). The background production of IFN-γ for the nanosphere group was 900 +/- 68 (24 h), 456 +/- 26 (48 h), and 1285 +/- 179 (72 h). Representative results from one of two experiments are shown.

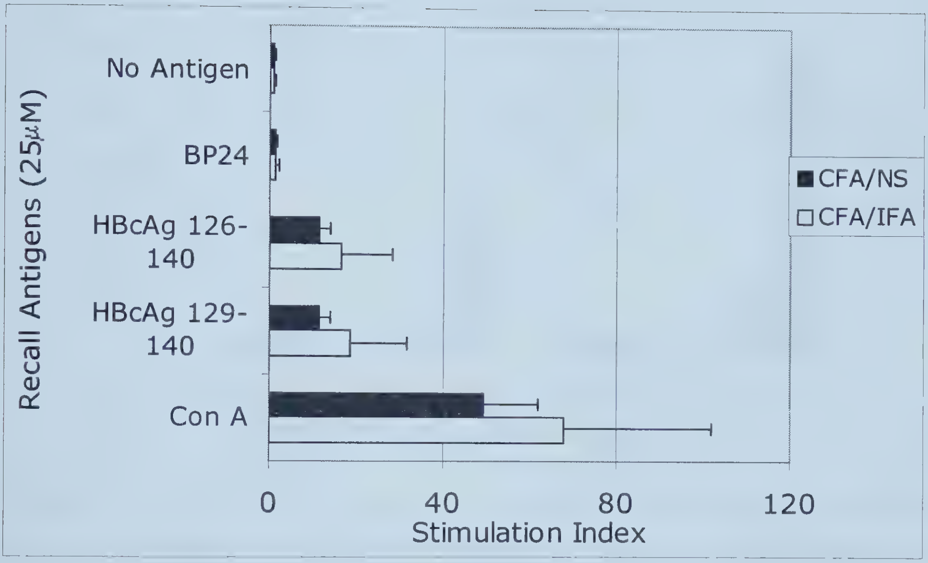

Figure 4-4. T cell proliferation after immunization of primed mice. Mice were primed with HBcAg$_{129\text{-}140}$ and CFA. Mice were then treated with HBcAg$_{129\text{-}140}$ either emulsified with IFA or encapsulated along with MPLA in PLGA (MW=6000) nanospheres. The proliferative response of T cells from immunized mice to a panel of recall antigens was measured by 3H-thymidine incorporation. Stimulation Index (SI) = cpm of test group/background cpm of wells without antigen. The background cpm for the CFA/IFA group was 383 +/- 120. The background cpm for the CFA/NS group was 2974 +/- 963. Representative results from one of two experiments are shown.

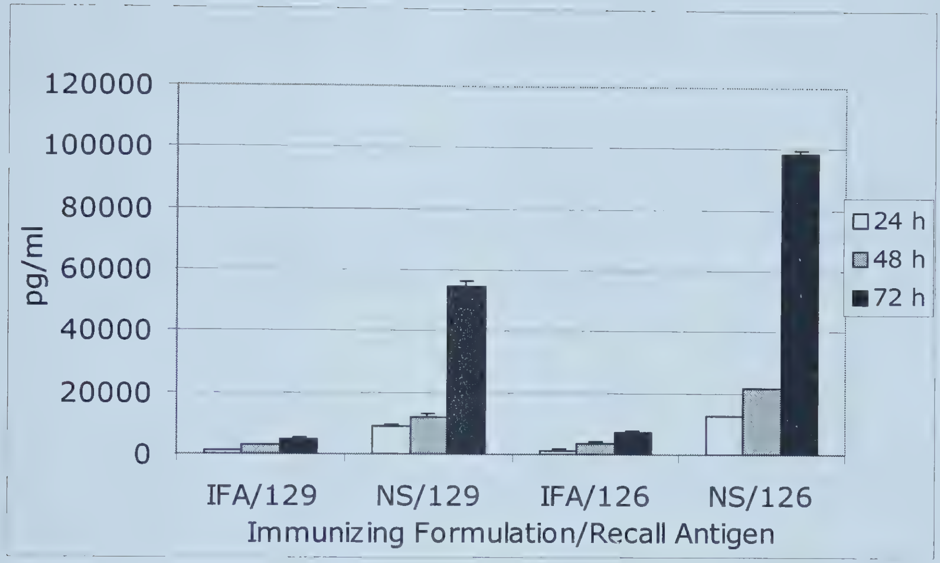

Figure 4-5. Interferon-γ production after immunization of primed mice. Mice were primed with $\text{HBcAg}_{129\text{-}140}$ and CFA. Mice were then treated with $\text{HBcAg}_{129\text{-}140}$ either emulsified with IFA or encapsulated along with MPLA in PLGA (MW=6000) nanospheres. The production of IFN-γ by T cells from immunized mice in response to recall antigen was measured by ELISA. Values in pg/ml were calculated using a standard curve of known quantities. Immunizing formulation: IFA=incomplete Freund's adjuvant and peptide, NS=nanosphere formulation of peptide. Recall antigen: 129= $\text{HBcAg}_{129\text{-}140}$, 126= $\text{HBcAg}_{126\text{-}140}$. The background production of IFN-γ for the IFA group was 678 +/- 145 pg/ml (24 h), 279 +/- 27 pg/ml (48h), and 1193 +/- 136 pg/ml (72 h). The background production of IFN-γ for the nanosphere group was 907 +/- 150 (24 h), 711 +/- 62 (48 h), and 718 +/- 30 (72 h). Representative results from one of two experiments are shown.

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Chapter Five

Analysis of Poly(D,L-lactic-co-glycolic acid) Nanosphere Uptake by Human Dendritic Cells and Macrophages *In vitro*

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5.1 Introduction

Antigen acquisition by dendritic cells (DC) is important in determining the outcome in viral infections and cancer, where Th1 responses are thought to be protective. Therefore, therapeutic vaccines for cancer and viral infections should be designed to deliver antigens to DC. Despite studies demonstrating the phagocytic ability of DC, phagocytosis of clinically relevant vaccine delivery systems by DC has not been systematically characterized. In this series of investigations, the phagocytosis of PLGA nanospheres by DC was confirmed and characterized. In addition, this phagocytosis was compared with particle uptake by macrophages (Mϕ).

5.2 Materials and Methods

5.2.1 Isolation of Mononuclear Cells from Peripheral Blood Leukocytes (PBLs)

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood leukocyte preparations collected from normal blood donors by Canadian Blood Services (Edmonton, Alberta, Canada) by density gradient separation over Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden). To remove residual platelets, the cells were washed with Hank's Balanced Salt Solution (HBSS) (BioWhittaker, Walkersville, MD, USA). PBMCs were depleted of T lymphocytes by rosetting with neuraminidase (Sigma Chemical Co., St. Louis, MO, USA) -treated sheep red blood cells (RAM Media, Calgary, Alberta, Canada). The T lymphocyte depleted (> 90%) mononuclear cells were isolated by density gradient centrifugation, washed in HBSS, and resuspended in RPMI-10HS [RPMI 1640 supplemented with 200 mM L-glutamine, 50 μg/ml gentamicin solution (Gibco-BRL, Burlington, Ontario, Canada) and 10% human serum (blood group AB; ICN, Costa Mesa, CA, USA)].

5.2.2 Culture of DC and Mϕ

T cell depleted mononuclear cells were plated in 6 well tissue culture plates (Corning 25815, Fisher Scientific, Nepean, Ontario, Canada) at 2 x 10^6 cells/well in RPMI-10HS. After incubation for 1 hour at 37°C/5% CO_2, non-adherent cells were removed by gentle washing and adherent cells were cultured with RPMI-10HS enriched with 500 U/ml GM-CSF (Pharmingen) and 8 ng/ml IL-4 (Pharmingen) to generate DC (1-3). For the generation of Mϕ, the adherent cells were cultured without IL-4. Culture media was replaced every 3 days.

5.2.3 Mixed Lymphocyte Reaction (MLR)

After 3 days in culture, DC and Mϕ were collected and washed 3 times with RPMI media before being resuspended to 1 x 10^5 cells/ml in RPMI-10HS media. T cells from an allogeneic donor were removed from –70°C storage, thawed, washed with RPMI media and resuspended at 1 x 10^6 cells/ml in RPMI-10HS. The MLR was set up at 3 different stimulator (S): effector (E) ratios: 1:5, 1:20, and 1:50 in triplicate in a 96 well plate. After 3 days at 37°C, 1 μci of ^{3}H-thymidine was added to each well in 50 μl media. After 24 hours the plate was harvested onto a filtermat using a Mach III Harvester 96 and the filter was read in a Microbeta Trilux reader (Wallac, Turku, Finland).

5.2.4 Monophosphoryl Lipid A (MPLA)

Synthetic MPLA was supplied by Biomira Inc.

5.2.5 Preparation of TMR-Dextran Encapsulated in PLGA Nanospheres

PLGA nanospheres were prepared using a water/oil/water solvent evaporation technique. Briefly, for the preparation of nanospheres containing MPLA and TMR, 100 μl of a 10 mg/ml solution of tetramethylrhodamine (TMR) conjugated dextran (Molecular Probes, Eugene, OR, USA) was emulsified in 300 μl of chloroform (Fisher Scientific, Nepean, ON, Canada) containing 100 mg of PLGA (BPI, Birmingham, AL, USA) polymer (lactic acid: glycolic acid ratio 50:50; MW=6000 or 50,000) and 200

μl of a 2 mg/ml solution of monophosphoryl lipid A (MPLA) (Biomira Inc, Edmonton, AB, Canada) using a micro tip sonicator (Model XL2010, Heat Systems INC., Farmingdale, NY, USA). For nanospheres without adjuvant, the TMR was emulsified in 500 μl of chloroform containing 100 mg of PLGA. The resulting primary emulsions were added into 2 ml of 9% w/v polyvinyl alcohol (PVA) (87-89% hydrolyzed, MW 31-50,000; Aldrich Chemical Co. Inc., Milwaukee, WI, USA) and was further sonicated to form the secondary emulsion. This emulsion was added drop wise to 8 ml of 9% w/v PVA and stirred for 3 h to evaporate the chloroform. The nanospheres were then collected by ultracentrifugation (40,000 x *g*, 10 minutes at 20°C), washed twice with distilled water (20 ml) to remove residual PVA, resuspended in 5 ml distilled water, and freeze-dried for 3 days. Nanospheres were stored at –20°C until needed.

5.2.6 Nanosphere Size Determination

Nanosphere size was determined by dynamic light scattering (model BI-90 Particle Sizer, Brookhaven Instruments Corp., Holtsville, NY, USA). The sample was diluted 100x with PBS, and size was measured at 25°C. TMR-dextran nanospheres measured 500 nm +/- 118 nm (mean +/- SD; n = 5).

5.2.7 Phagocytosis

On day 6, 7, or 8 of culture, 500 μg of PLGA nanospheres containing TMR-dextran (5 μg) with no adjuvant or with MPLA (2 μg) in 100 μl were added to wells. Control wells were pretreated for 10 minutes with 5 μg/ml of a phagocytosis inhibitor, cytochalasin B (4). Twenty four hours later cells were harvested, washed and resuspended in staining buffer. Phagocytosis was measured by flow cytometry.

5.2.8 Confocal Microscopy

On day 3 in culture, DC were transferred into Lab-Tek II 8 well chamber slides (Nalge Nunc Int., IL. USA) at a concentration of 2 x 10^5 cells/300 μL. Control wells were pretreated for 10 minutes with 5 μg/ml cytochalasin B

(Sigma-Aldrich Canada Ltd., Ontario, Canada). Each chamber received 100 μg of TMR-dextran containing nanospheres in 100 μl. After 24 hours the supernatant was removed and the cells were washed 3 times with 500 μl of PBS buffer. To label the cell membranes, the cells were incubated with 100 μl of 0.0005 % fluorescein-5-isothiocyanate (FITC)-concanvalin A (Molecular Probes, OR, USA) in PBS buffer for 2 minutes. Cells were washed 3 times with PBS and then were fixed with 100 μl of 4% paraformaldehyde in PBS for 10 minutes. After a final wash with PBS, slides were prepared with a solution of 2.5% 1,4-Diazabicyclo[2.2.2.]octane (Aldrich Chemical Company Ltd., WI, USA) and 7.5% gelatin in 50:50 PBS:glycerol. Slides were then examined by confocal microscopy using a Zeiss 510 LSM NLO (Carl Zeiss Microscope Systems, Zena, Germany).

5.2.9 Fluorescence Activated Cell Sorting (FACS)

After 24 h of incubation with TMR-dextran containing nanospheres, DC or Mϕ were harvested by scraping and washed. Aliquots of 2 x 10^5 cells were added into microcentrifuge tubes in 19 μl of cold wash buffer (PBS containing 10% fetal calf serum and 0.05% sodium azide). Mabs specific for CD14 (0.5 mg/ml), CD80 (1 mg/ml), and MHC II (0.5 mg/ml) were diluted five fold with buffer. Anti-CD86 was used without dilution at a concentration of 0.25 mg/ml. Two μl of mAb solution was added to each tube. After 30 minutes of incubation at 4°C, the cells were washed 3 times with wash buffer. Pellets were resuspended in 100 μl of wash buffer and 2 μl of FITC-conjugated secondary Ab (0.5 mg/ml) (either FITC-IgG1, FITC-IgG2a, or FITC-IgM) was added to each tube. After 30 minutes at 4°C in the dark, cells were washed with the wash buffer and transferred to Falcon tubes. Samples were analyzed by flow cytometry on a Becton-Dickinson FACScan (Franklin Lakes, NJ, USA).

The fluorescence threshold for cells described as positive for TMR (TMR^+) or FITC ($FITC^+$) was established as follows. Cells that were not treated with TMR nanospheres were used to set the threshold for 'red' fluorescence. Cells

treated with negative control FITC-antibody (isotype matched) were used to set the threshold for 'green' fluorescence. The baseline threshold was set so that greater than 98% of the negative cells fell below that value. There were slight day-to-day variations, but the threshold was always set just above 10^1 on the log scale measuring fluorescence intensity. Cells above this threshold level were considered to be positive for TMR or FITC.

5.3 Results

5.3.1 Characterization of Human Dendritic Cells and Macrophages

Parallel cultures of DC and Mϕ were generated using monocyte precursors from the same donor using GM-CSF and IL-4 (for DC) or GM-CSF alone (for Mϕ). This is a well established and widely used methodology for *in vitro* generation of DC and Mϕ (1-3). The identity of DC and Mϕ in cultures was confirmed by the presence of cell surface markers CD14, major histocompatibility class II molecules (MHC II), CD80, and CD86. On day 8 of culture, IL-4^+ cultures were CD$14^{-/low}$, MHC II$^+$, CD80^{low}, and CD86^{hi} and IL-4^- cultures were CD14^{hi}, MHC II$^+$, CD$80^{-/low}$, and CD$86^{-/low}$ (Figure 5-1). It is also possible to verify the identity of the cells as DC through a functional assay, the mixed lymphocyte reaction (MLR) (5). The MLR studies demonstrated that the DC culture was more efficient at stimulating the T cell response to allogeneic cells than the Mϕ culture ($p \leq 0.05$) (Figure 5-2). This functional assay confirms the identify of cells in the DC culture as DC.

5.3.2 Phagocytosis of PLGA Nanospheres by Human DC and Macrophages

PLGA nanospheres were visible inside DC after a 24 h co-incubation (Figure 5-3B and D), but nanospheres were not visible inside the DC when cells were pretreated with cytochalasin B, a phagocytosis inhibitor (Figure 5-3A and C).

It can be argued that phagocytosis in DC cultures is due to contaminating Mϕ. Two-colour staining with TMR-dextran encapsulated in nanospheres and

FITC-labeled antibodies to cell surface markers was used to unambiguously identify the phagocytic populations in the DC and Mϕ cell cultures. In order to identify the phagocytic subset of the cells, the cell surface marker expression on all live cells in culture was compared with the expression on TMR^+ cells.

CD14 is a monocyte marker constitutively expressed at high levels on Mϕ (6, 7). Some monocyte-derived DC express low levels of CD14 and this expression may increase during activation, for example, after phagocytosis or exposure to immunostimulatory agents (6). The phagocytic population in day 8 DC cultures had a significantly lower number of $CD14^+$ cells than in the Mϕ cultures (Figure 5-4A & B) ($p<0.005$). Mean fluorescence intensity of positive cells was 73 for Mϕ and 6 for DC. This lack of high CD14 expression on the TMR+ cells demonstrates that non-Mϕ cells in the DC culture are indeed phagocytic.

MHC Class II molecules are constitutively expressed by DC and Mϕ (8-11). Virtually all DC and Mϕ expressed some MHC II (data not shown). However, DC expressed higher levels of MHC II per cell than Mϕ (Figure 5-4C).

CD80 is a costimulatory molecule that is not constitutively expressed on DC, but is upregulated during maturation or under certain culture conditions. In this study, DC cultures had a higher proportion of cells expressing CD80 than Mϕ cultures (Figures 6A and B). The increased expression of CD80 on test cells as compared to control cells signifies that the marker was upregulated within the last 24 h in culture after the nanospheres were added. CD86 is another costimulatory molecule involved in the activation of T cells (8-11). There was a significant difference in the number of cells expressing CD86 between DC and Mϕ cultures (Figure 5-4A & B) ($p<0.0001$). Up to 90% of DC expressed CD86, while very few Mϕ expressed it.

5.3.3 DC and Mϕ Phagocytose PLGA Nanospheres to the Same Extent

DC and Mϕ were compared for their extent of phagocytosis of PLGA nanospheres. On day 8, nanospheres containing TMR-dextran were added to DC and Mϕ cultures. After 24 h both the number of phagocytic cells in culture (Figure 5-5A), as evidenced by the fraction of cells with a fluorescence signal, and the average amount of particle uptake per cell (Figure 5-5B), as indicated by the mean fluorescence intensity (MFI), were determined by flow cytometry. There were no significant differences between the two types of APC with regard to the number of phagocytic cells or the extent of phagocytosis per cell ($p \geq 0.05$). Control wells for both DC and Mϕ containing cytochalasin B were scarcely above the background fluorescence of unstained cells, confirming that the internalization of particles was due to phagocytosis but not adherence of nanospheres to the outside surface of the cells ($p<0.0001$).

5.3.4 Effect of Time in Culture on Phagocytosis

To determine the optimal day in culture for assessing phagocytosis of DC and Mϕ, the uptake of PLGA nanospheres over a 24 h period on days 6, 7, and 8 was examined. There was little or no difference in the number of phagocytic cells (Figure 5-6A) or the average fluorescence per cell (Figure 5-6B) between days 6, 7, or 8 in DC. For Mϕ there was also no significant difference in the number of phagocytic cells on different days in culture (Figure 5-6A). However, the number of particles per cell increased with increasing days in culture (Figure 5-6B).

5.3.5 Kinetics of Phagocytosis of PLGA Nanospheres

Day 8 cultures of DC and Mϕ were studied for the kinetics of phagocytosis. Cells were incubated with nanosphere formulations, collected at different time points and analyzed for the number of phagocytic cells and mean fluorescence per cell. No significant differences were seen at any time point between DC and Mϕ in terms of the number of phagocytic cells (Figure 5-7A)

or the amount of uptake per cell (Figure 5-7B). Incorporation of MPLA appeared to have no effect on the number of phagocytic cells at any time point (Figure 5-7A). However the number of particles per cells for the formulation containing MPLA was greater than that without any adjuvant (Figure 5-7B). Between 12 and 24 h, the number of phagocytic cells did not increase, but the MFI did (data not shown).

5.3.6 Effect of MPLA on Expression of Cell Surface Markers by DC

The effects of MPLA in the formulation on the expression of cell surface markers was examined on day 8 in cultures. MPLA had no significant effect on the expression of MHC II (Figure 5-4C), CD80 or CD 86 (Figure 5-4A).

5.4 Discussion

Antigen uptake by DC is a prerequisite for T cell mediated immune responses. Therefore, effective delivery of antigens to DC is of paramount significance to therapeutic vaccines designed to activate T cell responses. It has only recently been recognized that DC are phagocytic, and to date most phagocytosis studies have focused on microorganisms and on particles not suitable for human administration (12, 13). PLGA nanospheres are a useful antigen delivery system capable of formulating a variety of antigens for use in humans (14-18). The purpose of this set of investigations was to demonstrate that DC are capable of phagocytosing PLGA nanospheres, and to begin characterizing this phagocytosis.

Preliminary studies demonstrated qualitatively that DC could phagocytose PLGA nanospheres (Figure 5-3), but a method that could quantify phagocytosis was sought. Fluorescent nanospheres and flow cytometry were selected for several reasons. First, compared to other potential techniques, including the use of radiolabeled protein encapsulated in nanospheres, the methodology was simple. This method also eliminated the need for separating free nanospheres from the cells since the flow cytometer does this easily on the basis of size and granularity. In addition, the use of a flow

cytometer permitted the gathering of data on the number of phagocytic cells as well as the amount of particle uptake per cell. Other methods were unable to provide this level of detail. Finally the use of fluorescent nanospheres allowed the employment of dual colour staining to positively identify the phagocytic cell populations in culture on the basis of cell surface marker expression.

Parallel cultures of DC and Mϕ generated from the same individual were examined because this has not previously been done in studies of phagocytosis by human APC and it allowed us to look for similarities and differences between the scavenger Mϕ and the efficient antigen presenting cell, DC. The results demonstrated that DC phagocytose PLGA nanospheres to the same extent as Mϕ. This uptake of fluorescent particles is almost completely blocked by pretreatment with cytochalasin B, an agent that blocks phagocytosis, but not pinocytosis or normal metabolism in mammalian cells including human leukocytes (4). Thus, the uptake was due to particle phagocytosis and not due to pinocytosis of free TMR or to adherence of nanospheres to the outside of the cells. These results show that PLGA nanospheres are suitable for delivery of antigens to dendritic cells as well as macrophages.

Preliminary experiments demonstrated that maximal levels of phagocytosis are reached within 24 h in DC and Mϕ. Therefore, it was of interest to identify any differences between the cell types at earlier time points. Mϕ have been shown to be more efficient at particulate uptake (13); so it was important to determine if this was true for PLGA nanospheres. The experimental results demonstrate that there are no significant differences between the cells with regard to the rate of phagocytosis or the time required to reach the plateau of maximal phagocytosis. It was interesting to note that the number of phagocytic cells did not increase after 12 h, but the MFI (representing the number of particles per cell) continued to increase until 24 h. This indicates that all cells capable of phagocytosis of particles have taken up particles by

12 h, but that they continue to phagocytose additional particles for up to 12 more hours.

The maturation stage of DC affects their ability to phagocytose particles (13, 19). In order to determine the optimal timing for further experiments, the effect of time-in-culture on phagocytosis was examined. Phagocytosis of PLGA nanospheres by DC during a 24 h period was examined on days 6, 7, and 8 in culture. No difference was seen between days 6, 7, and 8 with respect to either the number of positive cells or the number of particles taken up per cell. Previous studies examining DC phenotype indicate that maturation occurs from day 9 in culture onwards (unpublished results), so phagocytosis may decrease after this point. In contrast, there was no difference in the number of phagocytic cells on different days in Mϕ cultures, but the number of particles taken up per cell seemed to increase as the time in culture increased.

It can be argued that phagocytosis in DC cultures is due to contaminating Mϕ. To eliminate this possibility, two-colour staining with TMR-dextran encapsulated in nanospheres and FITC-labeled antibodies to cell surface markers was used to identify the phagocytic populations in the DC and Mϕ cultures. The expression of cell surface markers on all live cells was compared with expression on the phagocytic population alone (TMR^{+} cells) to classify the phagocytic subset of cells in the cultures. For all surface markers studied, there were no differences in expression between all live cells and the phagocytic cells within the culture. The cells of the DC cultures were MHC II^{hi}, CD86^{hi}, CD80^{+}, and CD14$^{-/low}$, identifying them as DC rather than Mϕ. The cells of the Mϕ cultures were CD14^{hi}, MHC II^{+}, CD86^{low}, and CD80$^{-/low}$, confirming their identification as Mϕ.

MPLA is an adjuvant that aids in directing the immune response towards a T cell response. Because of its potential use in therapeutic vaccine formulations, it is important to understand what effects this adjuvant may

have on the acquisition of antigen by DC and the presentation of antigen by DC to T lymphocytes. The first step in studying these effects is to delineate any consequences of MPLA content on the phagocytosis of PLGA nanospheres. The results showed that the incorporation of MPLA into the formulation led to increased phagocytosis by DC at early time points, but that it may have negative consequences on phagocytosis after 24 h in culture. Previous studies with the Mϕ cell line J774A showed a negative impact of MPLA on phagocytosis at 24 h and later, but an enhancement of phagocytosis at earlier time points (14). This is a significant area for further investigation.

The influence of MPLA on the expression of cell surface molecules was also explored. The incorporation of an adjuvant into a vaccine formulation may enhance the immune response by altering the presentation of antigen to T cells or the stimulation of T cells by DC. Any change in the presentation of antigen to T cells is likely to be caused by a change in the expression of MHC II on the surface of the APC. Altered expression of the costimulatory molecules, CD80 and CD86, will affect the ability of the APC to stimulate T lymphocytes. The results demonstrated that the incorporation of MPLA did not affect the expression of MHC II molecules on day 8 in culture. At this point in culture, the expression of MHC II is quite high and it is possible that any difference is difficult to discern. Preliminary data on the effect of MPLA on the expression of cell surface markers at earlier times in culture have been collected. These data suggest that MPLA may influence the maturation state of the cells as well as their ability to present antigen and stimulate T cells. Further investigations will clarify how to formulate antigen for achieving peak immune activation.

These investigations conclusively demonstrate that DC phagocytose PLGA nanospheres. The phagocytic population was unambiguously identified as DC based on expression of relevant cell surface markers. Since DC are the key professional antigen presenting cells capable of stimulating naïve T cells, the

data support the view that PLGA nanospheres can serve as an efficient delivery system for vaccines designed to activate T cell-mediated immune responses. This finding has significant implications for the development of therapeutic vaccines for cancer and chronic viral infections.

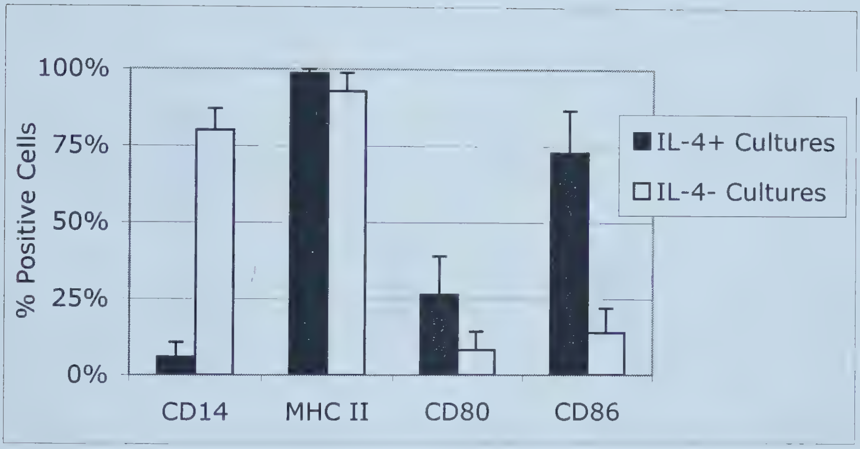


Figure 5-1. Characterization of human monocyte-derived cells in culture. Percentage of cells expressing cell surface markers, based on reactivity with specific antibodies. The data are presented as the mean +/- SD (error bars) of the values of the samples from 8 different donors.

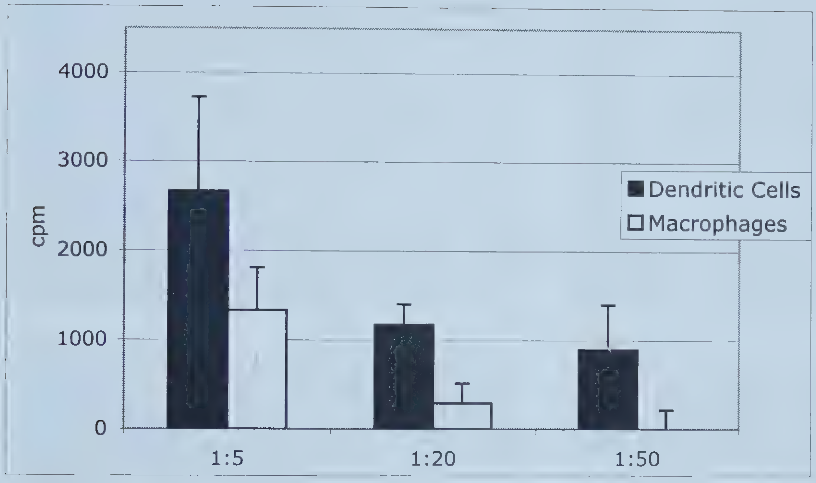


Figure 5-2. Activation of allogeneic T lymphocytes by dendritic cells and macrophages in culture (mixed lymphocyte reaction). DCs and Mϕ were incubated with T cells from an unrelated donor at varying stimulator (DC or Mϕ) to effector (T cells) ratios. The proliferation of the T cells was measured by incorporation of 3H-thymidine. The experiment was performed in triplicate using DC or Mϕ from one donor and the results are shown as mean cpm +/- SD (error bars).

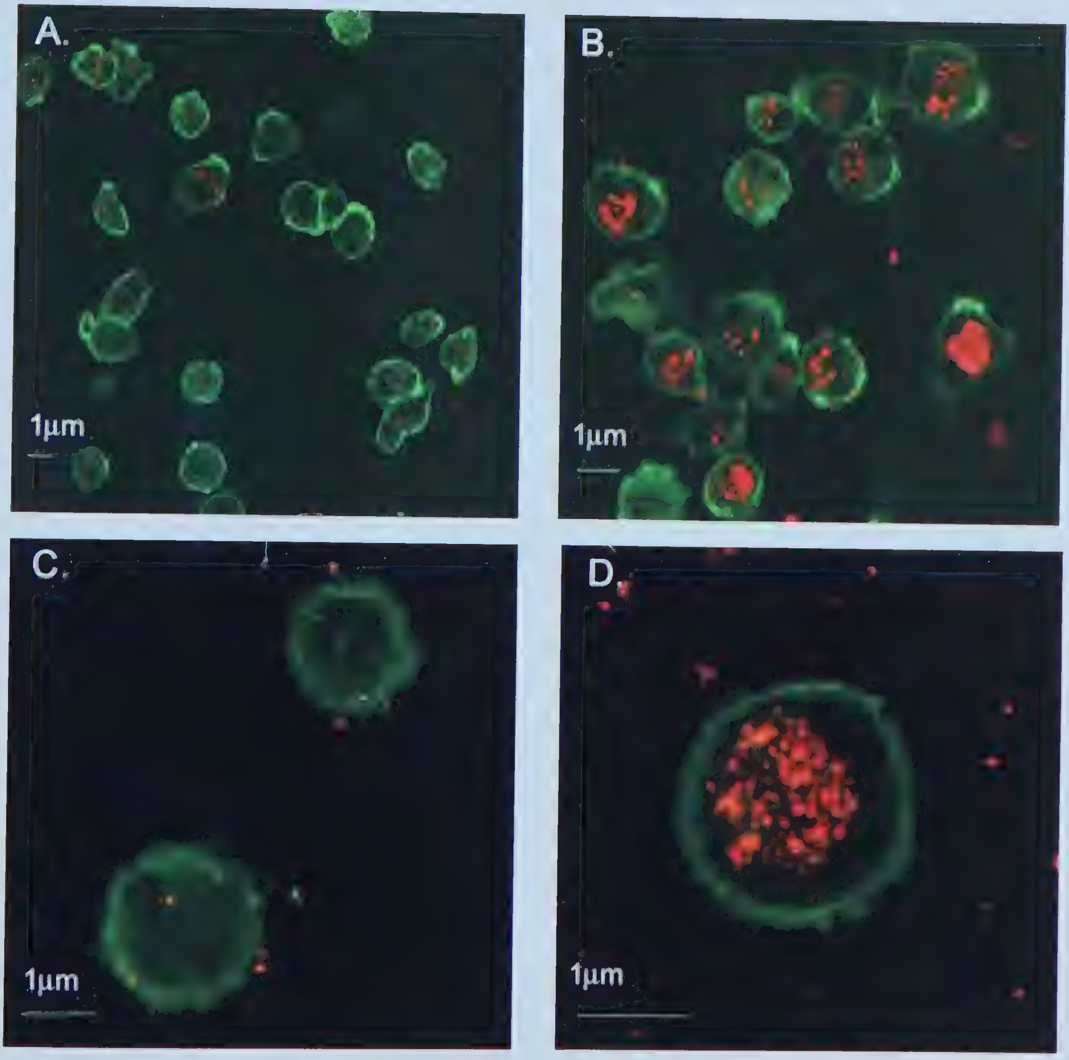


Figure 5-3. Uptake of PLGA nanospheres by human dendritic cells. On day 4, after 24 h incubation with TMR-dextran containing nanospheres, cell membranes were stained with FITC-labeled Concanvalin A and examined using a confocal microscope. Note the veiling on the cell membranes, which is a morphological trait of DC. Control cells (A and C) pretreated with cytochalasin B. Test cells (B & D) were untreated.

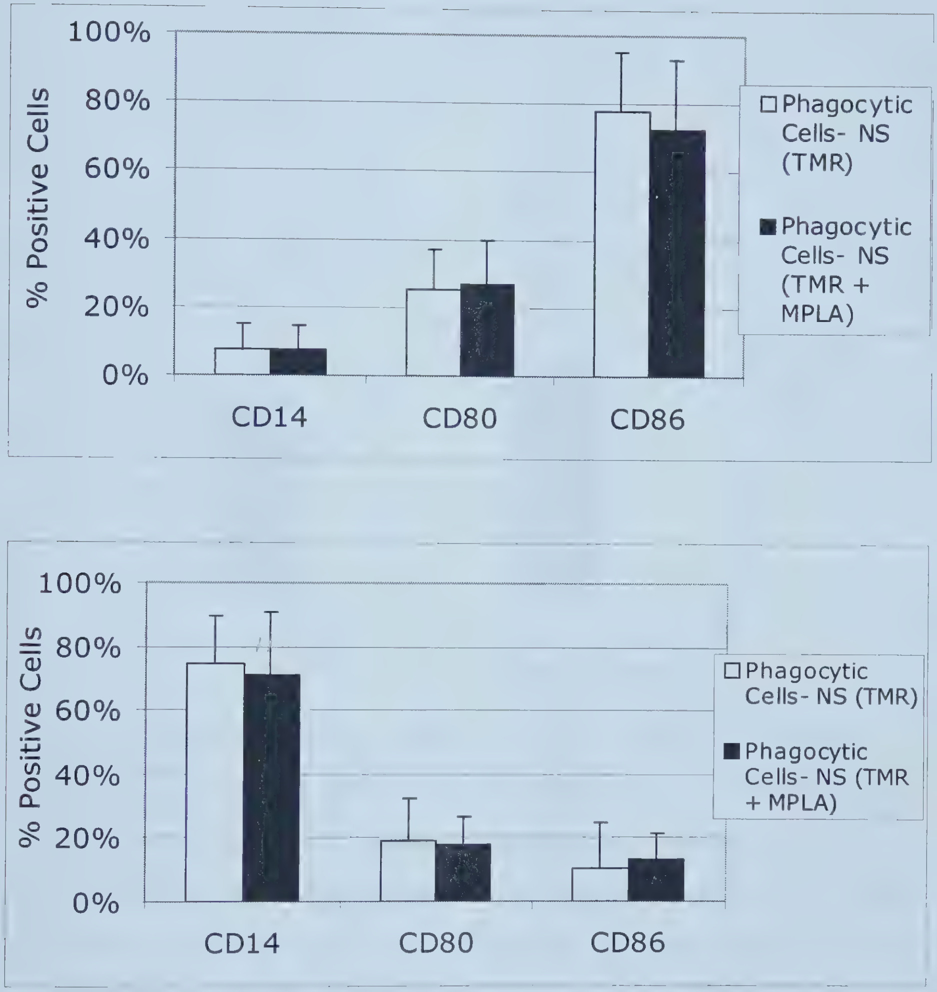


Figure 5-4 (A-B). Day 8 expression of cell surface markers on phagocytic dendritic cells and macrophages. On day 8, after phagocytosis of PLGA nanospheres containing TMR-dextran for 24 h, two-colour flow cytometry was used to determine the cell surface marker expression on phagocytic (i.e., TMR$^+$) cells. (A) DC expression of CD14, CD80, and CD86 represented as the percentage of cells positive for FITC-labeled antibody. (B) Mϕ expression of CD14, CD80, and CD86 represented as the percentage of cells positive for FITC-labeled antibody.

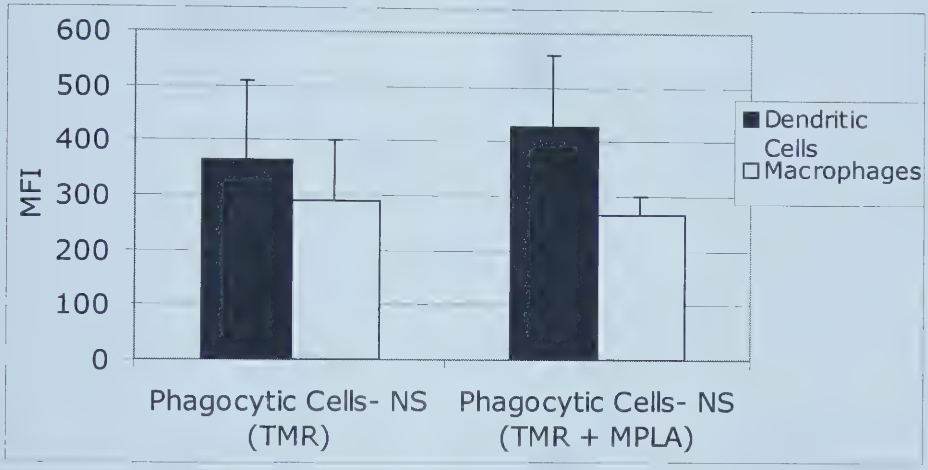
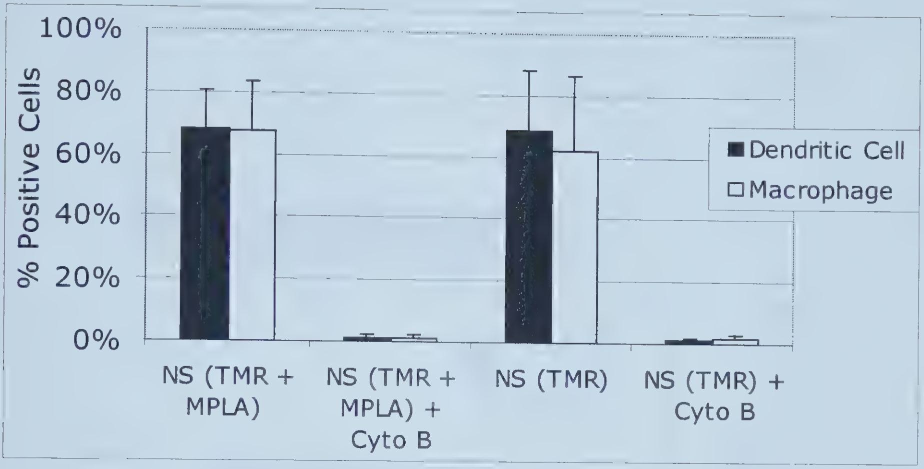


Figure 5-4 (C). Day 8 expression of cell surface markers on phagocytic dendritic cells and macrophages. On day 8, after phagocytosis of PLGA nanospheres containing TMR-dextran for 24 h, two-colour flow cytometry was used to determine the cell surface marker expression on phagocytic (i.e., TMR$^+$) cells. (C) Level of MHC II on DC and Mϕ represented as the average amount of fluorescence per cell. MFI is mean fluorescence intensity of the FITC signal. The data are presented as the mean+/- SD (error bars) of the values of the samples from 6 different donors.



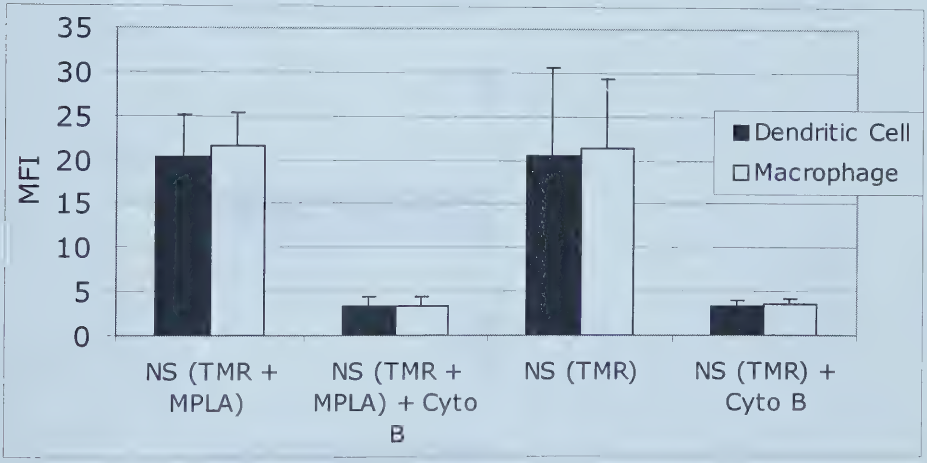


Figure 5-5. Dendritic cells and macrophages phagocytose PLGA nanospheres to the same extent. On day 8, phagocytosis of PLGA nanospheres containing TMR-dextran by DC and Mϕ over 24 h was measured. Control wells were pretreated with cytochalasin B. (A) The number of phagocytic cells in culture is expressed as the percentage of cells containing TMR. (B) The extent of phagocytosis was measured as the mean fluorescence intensity/cell (MFI). The data are presented as the mean+/- SD (error bars) of the values of the samples from 7 different donors.

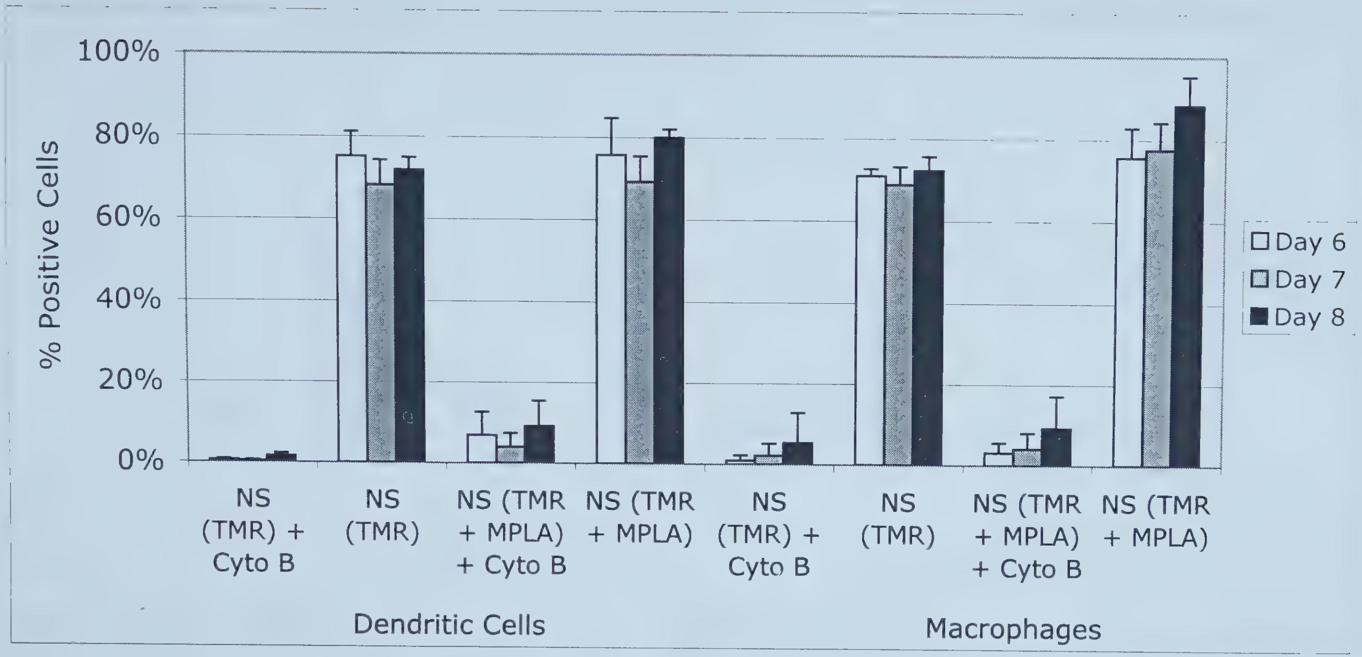


Figure 5-6 (A). Effect of time in culture on phagocytosis. On day 6, 7, and 8, phagocytosis over 24 h was measured in DC and Mϕ. (A) The number of phagocytic cells in culture is expressed as the percentage of cells containing TMR. The data are presented as the mean+/- SD (error bars) of the values of the samples from 3 different donors.

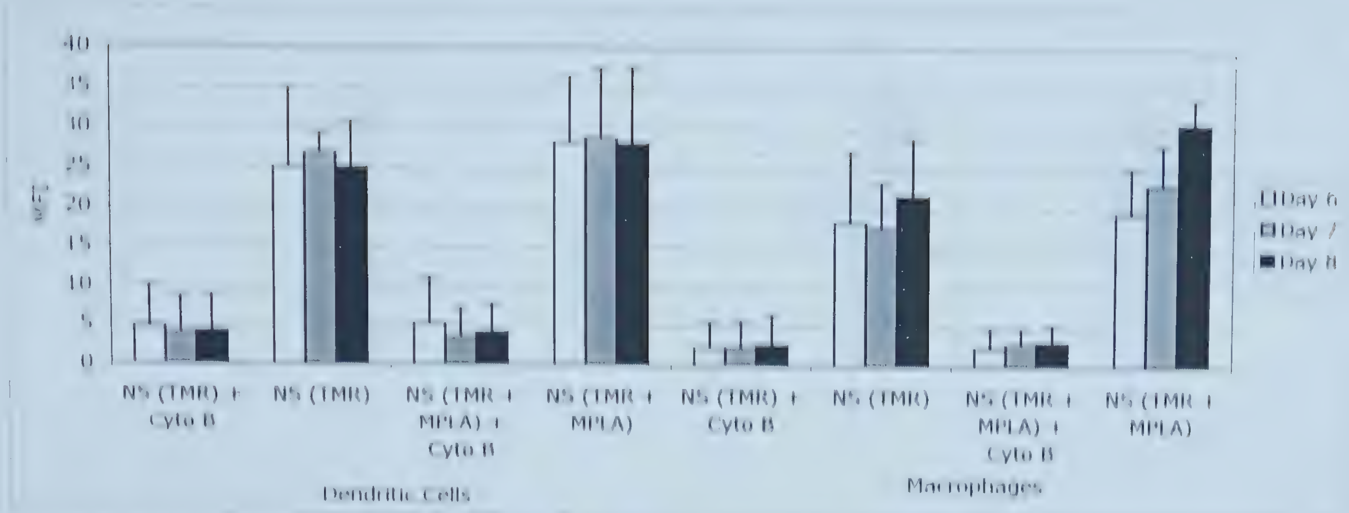


Figure 5-6 (B). Effect of time in culture on phagocytosis. On day 6, 7, and 8, phagocytosis over 24 h was measured in DC and Mϕ. (B) The extent of phagocytosis was measured as the mean fluorescence intensity/cell (MFI). The data are presented as the mean+/- SD (error bars) of the values of the samples from 3 different donors.

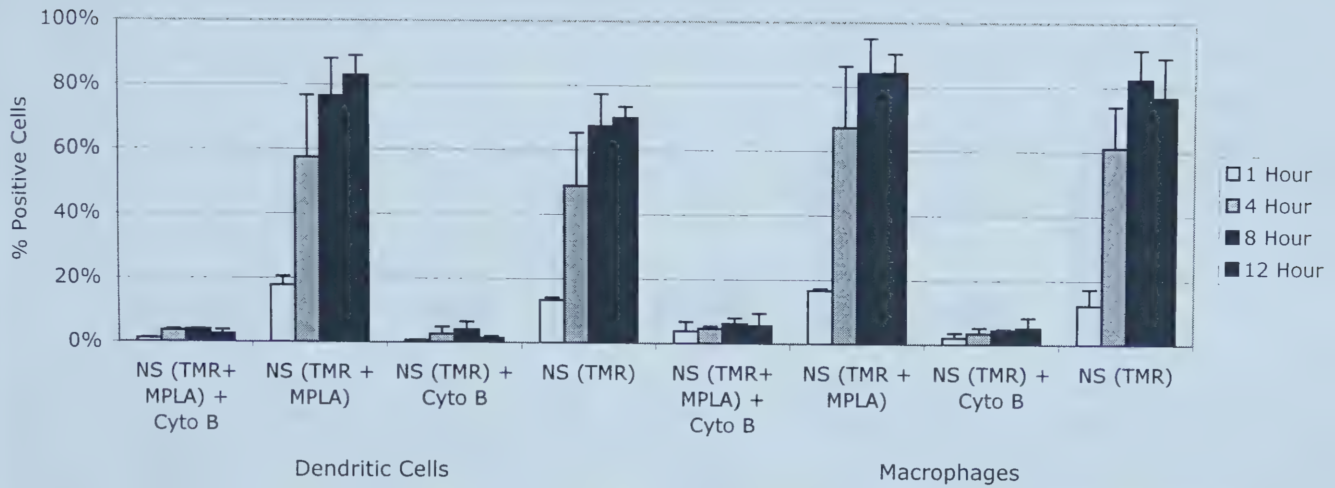


Figure 5-7 (A). Kinetics of phagocytosis of PLGA nanospheres. On day 8, phagocytosis over 1, 4, 8, and 12 hours was measured in DC and Mϕ. (A) The number of phagocytic cells in culture is expressed as the percentage of cells containing TMR. The data are presented as the mean+/- SD (error bars) of the values of the samples from 2 different

donors.

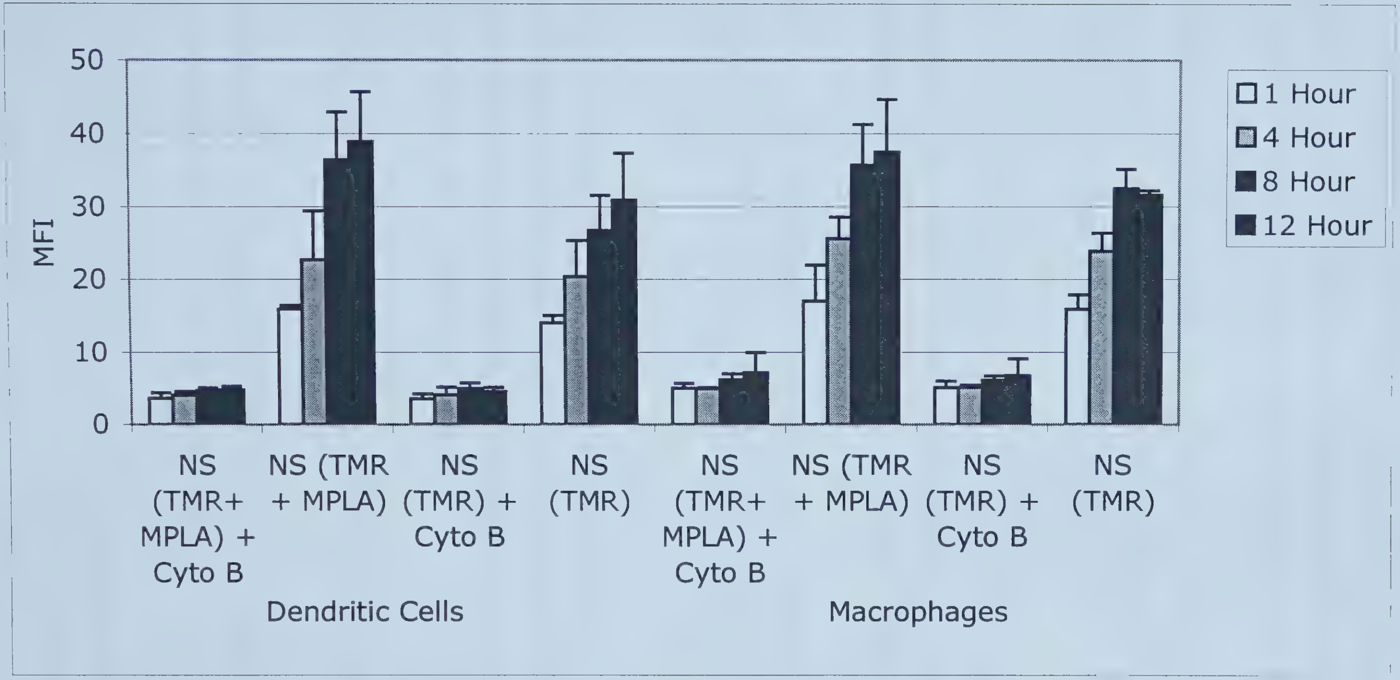


Figure 5-7 (B). Kinetics of phagocytosis of PLGA nanospheres. On day 8, phagocytosis over 1, 4, 8, and 12 hours was measured in DC and Mϕ. (B) The extent of phagocytosis was measured as the mean fluorescence intensity/cell (MFI). The data are presented as the mean+/- SD (error bars) of the values of the samples from 2 different donors.

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Chapter Six

Biodegradable Nanosphere Delivery of a MUC1 Lipopeptide Enhances Antigen Presentation to T Lymphocytes and Overcomes Self-tolerance

6.1 Introduction

BLP25, a lipopeptide corresponding the tandem repeat region of MUC1 mucin, is a strong candidate for the immunotherapy of $MUC1^+$ cancers. However, MUC1 is a self-antigen and tolerance to the antigen may prevent a therapeutic immune response. Dendritic cells (DC) are extremely powerful antigen presenting cells (APC) (1) and it is likely that their superior T cell stimulatory activity can be exploited to overcome tolerance to self-antigens. It has been shown that DC that have been pulsed *ex vivo* with peptide antigens or transduced with RNA specific for tumour antigens can, upon reinfusion, induce a strong tumour-specific CTL response and tumour regression (2-4). However, the *ex vivo* expansion of DC followed by reinfusion of the activated, peptide-pulsed cells into the patient is impractical for use in large numbers of patients. It is necessary for the purposes of developing expedient immunotherapeutic treatments to establish a method of delivering tumour antigens to the DC *in vivo*.

There are three primary issues to be considered when delivering tumour-specific antigen to DC *in vivo* for a T cell response. Firstly, soluble antigens, particularly peptides, are rapidly degraded *in vivo*. In addition, peptides are poorly immunogenic. Finally, phagocytosis of antigen leads to more effective presentation than pinocytosis of soluble antigen, likely due to the effective targeting of the antigen to the processing and presenting pathways in the APC (5). Each of these concerns can be addressed by formulating the antigen in a particulate antigen delivery system such as poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres.

The *in vitro* characterization of the immune response of human T cells to the nanosphere formulation containing BLP25 is necessary for several reasons. Because BLP25 is not a self-antigen in mice and tolerance is not a factor in that model, the response of human T cells to the vaccine formulation needs to be examined. There are obvious limits on *in vivo* human testing, but *in vitro* systems can easily be used to compare different formulations, to screen

panels of peptides for the ability to activate the immune system, and as a procedure to confirm that a formulation delivers antigen to DC in a manner that provokes a T cell response.

The purpose of this series of investigations was to demonstrate that, not only is the PLGA nanosphere formulation taken up by DC, but the incorporated antigen is presented to T cells in a manner that elicits a strong T cell proliferative response after a single stimulation. In addition, the effect of antigen delivery to DC on the expression of cell surface markers involved in the activation of T cells was examined.

6.2 Materials and Methods

6.2.1 Isolation of Mononuclear Cells from Whole Blood

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood leukocyte preparations collected from normal blood donors by Canadian Blood Services (Edmonton, Alberta, Canada) by density gradient separation over Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden). To remove residual platelets, the cells were washed with Hank's Balanced Salt Solution (HBSS) (BioWhittaker, Walkersville, MD, USA). PBMCs were depleted of T lymphocytes by rosetting with neuraminidase (Sigma Chemical Co., St. Louis, MO, USA) -treated sheep red blood cells (RAM Media, Calgary, Alberta, Canada). The T lymphocyte depleted (> 90%) mononuclear cells were isolated by density gradient centrifugation, washed in HBSS, and resuspended in RPMI-10HS [RPMI 1640 supplemented with 200 mM L-glutamine, 50 μg/ml gentamicin solution (Gibco-BRL, Burlington, Ontario, Canada) and 10% human serum (blood group AB; ICN, Costa Mesa, CA, USA)].

6.2.2 Culture of DC

T cell depleted mononuclear cells were plated in 6 well tissue culture plates (Corning 25815, Fisher Scientific, Nepean, Ontario, Canada) at 2 x 10^6 cells/well in RPMI-10HS. After incubation for 1 hour at 37°C/5% CO_2, non-

adherent cells were removed by gentle washing and adherent cells were cultured with RPMI-10HS enriched with 500 U/ml GM-CSF (Pharmingen) and 8 ng/ml IL-4 (Pharmingen) to generate DC.

6.2.3 Collection of T Lymphocytes

After separation of rosetted T cells from the PBMCs, the pellet containing sheep RBCs and T lymphocytes was lysed with 15 mL ACK lysis buffer. T cells were washed three times with HBSS, resuspended to 1 x10^7 cells/ml in RPMI-10HS, and frozen for storage at –70°C in 10% DMSO.

6.2.4 Monophosphoryl Lipid A (MPLA)

Synthetic MPLA (MPLA-B) was supplied by Biomira Inc. (Edmonton, AB, Canada). MPLA-A, MPLA produced by chemical modification of lipid A, was obtained from Avanti Lipids (Alabaster, AL, USA).

6.2.5 BLP25

The lipopeptide with the sequence STAPPAHGVSTAPDTRPAPGSTAPP-Lys(Palmitoyl) was supplied by Biomira Inc. (Edmonton, AB, Canada).

6.2.6 Preparation of PLGA Nanospheres

PLGA nanospheres containingBLP25 were prepared using a single emulsion solvent evaporation technique while nanospheres containing tetramethylrhodhamine (TMR) conjugated to dextran were prepared using a double emulsion technique (6).

For nanospheres containing BLP25 without adjuvant, 100 μL of a 1 mg/ml solution of BLP25 was added to 400 μl of chloroform containing 100 mg of PLGA (BPI, Birmingham, AL, USA) (MW=6,000). For nanospheres containing adjuvant alone, 200 μl of a 2 mg/ml solution of MPLA was added to 300 μl of chloroform containing 100 mg of PLGA. For nanospheres containing both

peptide and adjuvant, 100 μL of a 1 mg/ml solution of BLP25 and 200 μl of a 2 mg/ml solution of MPLA were added to 200 μl of chloroform containing 100 mg of PLGA. All formulations were emulsified with 2 ml of 9% w/v polyvinyl alcohol (PVA) (87-89% hydrolyzed, MW 31-50,000; Aldrich Chemical Co. Inc., Milwaukee, WI, USA) using a microtip sonicator.

For nanospheres containing TMR-dextran, 100 μl of a 10 mg/ml solution of TMR conjugated dextran (Molecular Probes, Eugene, OR, USA) was emulsified in 500 μl of chloroform containing 100 mg of PLGA (BPI, Birmingham, AL, USA) polymer (MW=50,000) with no adjuvant or with 200 μl of a 2 mg/ml solution of MPLA-A or MPLA-B using a microtip sonicator (Model XL2010, Heat Systems INC., Farmingdale, NY, USA). All formulations were emulsified using a microtip sonicator. To each emulsion was added 2 ml of 9% w/v PVA and the formulations were again emulsified using a microtip soniocator.

All final emulsions were added drop-wise into 8 ml of 9% w/v PVA and stirred for 3 h to evaporate the chloroform. The nanospheres were then collected by ultracentrifugation (40,000 x *g*, 10 minutes at 20°C), washed twice with distilled water (20 ml) to remove residual PVA, resuspended in 5 ml distilled water, and freeze-dried for 3 days. Nanospheres were stored at –20°C until needed. Prior to their addition to culture, nanospheres were resuspended in RPMI-10HS at a concentration of 5 mg/ml.

6.2.7 Nanosphere Size Determination

Nanosphere size was determined by dynamic light scattering (model BI-90 Particle Sizer, Brookhaven Instruments Corp., Holtsville, NY). The sample was diluted 100x with PBS, and size was measured at 25°C. BLP25 nanospheres measured 290 +/- 86 nm (mean +/- SD; n=3). TMR-dextran nanospheres measured 500 nm +/- 118 nm (mean +/- SD; n = 5).

6.2.8 Phagocytosis

On day 3 of culture, 500 μg of PLGA nanospheres containing TMR-dextran (5 μg) with no adjuvant or with MPLA (2 μg) in 100 μl were added to wells. Control wells were pretreated with 5 μg/ml of a phagocytosis inhibitor, cytochalasin B (Sigma Chemical Co., St. Louis, MO, USA) (7). Twenty four hours later the cells were harvested, washed and resuspended in cold wash buffer (PBS containing 10% fetal calf serum and 0.05% sodium azide). Phagocytosis was measured by flow cytometry.

6.2.9 Fluorescence Activated Cell Sorting (FACS)

After 24 h of incubation with TMR-dextran containing nanospheres, DC or Mϕ were harvested by scraping and washed. Aliquots of 2 x 10^5 cells were added into microcentrifuge tubes in 19 μl of cold wash buffer (PBS containing 10% fetal calf serum and 0.05% sodium azide). Mabs specific for CD14 (0.5 mg/ml), CD80 (1 mg/ml), and MHC II (0.5 mg/ml) were diluted five fold with buffer. Anti-CD86 was used without dilution at a concentration of 0.25 mg/ml. Two μl of mAb solution was added to each tube. After 30 minutes of incubation at 4°C, the cells were washed 3 times with wash buffer. Pellets were resuspended in 100 μl of wash buffer and 2 μl of FITC-conjugated secondary Ab (0.5 mg/ml) (either FITC-IgG1, FITC-IgG2a, or FITC-IgM) was added to each tube. After 30 minutes at 4°C in the dark, cells were washed with the wash buffer and transferred to Falcon tubes. Samples were analyzed by flow cytometry on a Becton-Dickinson FACScan (Franklin Lakes, NJ, USA).

The fluorescence threshold for cells described as positive for TMR (TMR^+) or FITC ($FITC^+$) was established as follows. Cells that were not treated with TMR nanospheres were used to set the threshold for 'red' fluorescence. Cells treated with negative control FITC-antibody (isotype matched) were used to set the threshold for 'green' fluorescence. The baseline threshold was set so that greater than 98% of the negative cells fell below that value. There were

slight day-to-day variations, but the threshold was always set just above 10^1 on the log scale measuring fluorescence intensity. Cells above this threshold level were considered to be positive for TMR or FITC.

6.2.10 T Cell Proliferation

On day 3 in culture, the formulations were added to the DC. Five hundred micrograms of nanospheres were added, in 100 μl of media, to the appropriate wells. Fifty microliters of a 2 mg/ml solution of soluble antigen was added to the pertinent wells. On day 4, after 24 h of incubation with the formulations, DC were harvested by scraping and washed three times with RPMI-10HS. After resuspension of the DC, soluble antigen was replaced where necessary. T lymphocytes were thawed, washed with RPMI-10HS and resuspended to 1×10^6 cells/ml. The T cell proliferations were set up in 96 well plates. Each well contained 1×10^5 T cells + either 1×10^4 DC (1:10 S:E ratio) or 5×10^3 DC (1:20 S:E ratio) in 250 μl media. After 5-7 days at 37°C, 1 μci of ^{3}H-thymidine was added to each well in 50 μl media. The plate was harvested 24 hours later onto a filter mat using a Mach III Harvester 96 machine and the filter was read in a Microbeta Trilux reader (Wallac, Turku, Finland).

6.3 Results

6.3.1 Phagocytosis of PLGA Nanospheres by Immature DC

Previous studies have shown that PLGA nanospheres are efficiently phagocytosed by human DC and therefore may be suitable for enhanced delivery of antigens to DC (8). This was demonstrated using DC generated by culture of monocytes *in vitro* with GM-CSF and IL-4 for a period of 8 days. In the present study the phagocytosis of nanospheres was examined at an earlier time point, day 3, so as to reduce the DC culture period before antigen loading. Previous experience demonstrated that, at day 3 in culture, monocyte precursors are just beginning to exhibit a DC phenotype. Particulate uptake by DC is highly dependent on the maturation stage of the

cells; immature cells are highly phagocytic while fully mature cells lose the ability to phagocytose. In view of this, the uptake PLGA by the early DC or DC precursors on day 3 cultures was explored.

On day 3, nanospheres containing TMR-dextran were added to DC cultures. After 24 h, both the number of phagocytic cells in culture (Figure 6-1A), as evidenced by the fraction of cells with a fluorescence signal, and the average amount of particle uptake per cell (Figure 6-1B), as indicated by the mean fluorescence intensity (MFI), were determined by flow cytometry. Anywhere from 19-37% of the DC populations phagocytosed particles (Figure 6-1A). Wells treated with cytochalasin B contained the same number of fluorescent cells as the unstained control wells, confirming that the internalization of particles in test cells was due to phagocytosis (7). The proportion of phagocytic cells in day 3 cultures was significantly lower than that previously reported for day 8 cultures (> 80%) (8). This may be due to the fact that the majority of the precursor cells have not fully differentiated into dendritic cells at day 3. Fewer phagocytic cells were detected in the DC populations that received nanospheres containing either MPLA-A or MPLA-B than in the DC populations that received nanospheres without MPLA. This confirms previous results, which showed that while MPLA enhanced phagocytosis within the initial 12 h period, its effect of phagocytosis over 24 hour period was somewhat inhibitory (8).

6.3.2 Effect of Phagocytosis on Expression of Cell Surface Markers

In order to activate a T cell response, DC must express class II human leukocyte antigens (HLA II) molecules and costimulatory molecules such as CD80 and CD86. The expression of these markers on day 4 DC cultures that were either pre-incubated with PLGA nanospheres or untreated was examined.

HLA II molecules present peptide antigen to CD4+ T lymphocytes. Virtually all DC in culture express HLA II (data not shown). A comparison of the level

of expression of HLA II on the surface of the phagocytic cells (TMR$^+$) with that on all live cells in culture (TMR$^+$ and TMR$^-$) or untreated cells (Figure 6-2A) shows that phagocytosis of PLGA nanospheres leads to upregulation of HLA II on the surface of the cells. The type of formulation appears to affect HLA II expression; the two formulations containing MPLA demonstrated an elevated level of HLA II in comparison to DC that phagocytosed nanospheres that did not contain adjuvant.

CD80 and CD86 are costimulatory molecules necessary for the activation of T cells. CD80 was expressed on about 30% of all DC in culture at day 4 (Figure 6-2B) while CD86 was found on over 75% of all DC (Figure 6-2C). There was no difference seen in the proportion of cells expressing CD80 or CD86 between phagocytic and nonphagocytic cells. When the level of expression of CD80 was examined, there was no difference seen between phagocytic and nonphagocytic cells (data not shown), however, this was not the case for CD86. CD86 was expressed at higher levels on cells that had taken up PLGA nanospheres than on cells that were not incubated with nanospheres (Figure 6-2D). MPLA-A appeared to have a beneficial effect on the expression of CD86 in one individual but this effect was not seen in the other individual tested.

6.3.3 T Cell Activation by Antigen-loaded DCs

Now that it is confirmed that DC can phagocytose PLGA nanospheres and that they express HLA II, CD80, and CD86, molecules required for T cell activation, it remained to be shown that DC can process and present antigen from PLGA nanospheres to T cells in such a way as to break self-tolerance and elicit a T cell response to a self-antigen, BLP25.

In all five subjects, a strong proliferation was observed in response to DC that had taken up nanospheres containing both BLP25 and either MPLA-A or MPLA-B (Figures 3A-E). No significant proliferation was seen when the DC

were exposed to soluble BLP25, nanospheres containing BLP25 or adjuvant alone, or nanospheres containing adjuvant mixed with soluble BLP25.

There are individual differences in the strength of the T cell response. This is likely due to varying abilities of people of different genetic backgrounds to present the peptide, BLP25, on their HLA molecules as well as diverse numbers of naïve or primed T cells capable of responding to the antigen.

BLP25 is a peptide from human MUC1, which is a self-antigen in the experimental subjects. The proliferation seen in response to the formulation demonstrates the breaking of tolerance against this self-antigen.

6.4 Discussion

PLGA nanospheres can be phagocytosed by human DC *in vitro* on day 8 in culture (8). In this set of experiments particulate uptake by DC was examined at an earlier time in culture, day 3. It was demonstrated that DC are phagocytic at this earlier time point, however, there are less phagocytic cells in the culture than there are at day 8. This can be attributed to the lack of full differentiation of monocyte precursors into DC at this time point. It is also possible that cells that have taken up extremely low numbers of nanospheres are not detected as TMR$^+$, since TMR is not an optimal fluorochrome for flow cytometry and only 10% of the signal is detected.

Uptake of PLGA nanospheres did not affect the number of cells expressing relevant cell surface markers, but it did enhance the level of expression of both HLA Class II and CD86 on DC. This is in contrast to earlier studies using day 9 DC cultures, which demonstrated that there was no upregulation of cell surface markers after particulate uptake, regardless of the formulation used (8). The probable explanation for this discrepancy is that there is a gradual maturation of the DC caused by culture conditions and at day 8 the cells have already upregulated HLA Class II and CD86 in response to this. Future work exploring the effect of formulations on cell surface marker

expression should be performed at multiple time points to separate out the effects of *in vitro* manipulation from formulation effects.

The incorporation of adjuvant in the nanosphere formulation led to a greater increase in HLA II expression than was caused by nanospheres without adjuvant. The effect of adjuvant incorporation on CD86 expression was variable in the two individuals tested. Phagocytosis of PLGA nanospheres causes activation of DC into an antigen presenting phenotype and MPLA may offer an advantage in this area.

MUC1 is a strong candidate for immunotherapy of MUC1$^+$ tumours. Even in the absence of any immunotherapy, cancer patients may demonstrate an immune response against MUC1. Humoral responses against MUC1 can be seen in patients with breast, ovarian, colon, or pancreatic cancers (9-11). In addition, MUC1-specific CTL have been isolated from the tumour draining lymph nodes of breast (12) and ovarian cancer patients. Furthermore, in patients with breast, ovarian, and pancreatic adenocarcinomas, CTL specific for the tandem repeat portion of the MUC1 molecule can be induced *in vitro* (13-14). To date no MUC1-specific Th responses have been observed in cancer patients. It is likely that this lack of CD4$^+$ T cell help is responsible for the inability of patients to establish a more robust immune response and overcome the disease (15).

In vitro studies have demonstrated a proliferative response against MUC1 and MUC1-derived peptides in PBMCs from patients with ovarian adenocarcinoma (16). MUC1-specific Th cell responses have been generated *in vitro* by priming CD4$^+$ T cells with DC loaded with unglycosylated synthetic MUC1 peptide (17-18). The high concentration of peptides required to elicit a response suggests that, in addition to ineffective antigen processing, CD4$^+$ T cell tolerance may also contribute to the lack of MUC1-specific Th cells *in vivo* (18). BLP25 incorporated into liposomes has been shown to stimulate a potent anti-MUC1 T cell proliferation in PBL *in vitro* along with a class I-

restricted CTL response (19). These studies support the use of MUC1-derived peptides as immunotherapeutic agents for cancer, in the event that immune responses can be elicited *in vivo*.

Several studies have investigated the development of immune responses *in vivo* after immunization with MUC1-derived peptides. Patients immunized with MUC1 tandem repeats have been shown to develop high levels of MUC1-specific IgG and IgM (20-22). The evidence for the development of MUC1-specific T cell responses induced *in vivo* after vaccination in cancer patients is more controversial (23-25). Immunization of six cancer patients with a 106 amino acid MUC1 peptide conjugated to KLH along with QS-21 elicited strong IgG and IgM reactivity against the peptide but failed to demonstrate consistent T cell responses against MUC1 (26). In other studies, immunization with the 105 amino acid peptide and BCG, 5 variable number tandem repeats conjugated to mannan, or a 16 amino acid MUC1 peptide conjugated to KLH, elicited inconsistent T cell proliferation and CTL responses (23-25).

Mice transgenic for MUC1 have proved to be a useful tool in studying the immune response against MUC1 in a system that includes tolerance against the antigen, similar to that seen in cancer patients (27). In one study, fused DC and MUC1-expressing tumour cells were injected into transgenic mice and led to the establishment of both humoral and cell mediated immunity along with rejection of established metastases (28). Injection of irradiated tumour cells without fusion with DC did not lead to an immune response or to tumour rejection (28). Another set of investigations compared the immune responses seen after vaccination of transgenic mice with several formulations of a 140 amino acid MUC1-derived peptide. Mice injected with peptide and GM-CSF or peptide with SB-AS2 (an adjuvant containing MPLA and Quil A) developed strong humoral responses against MUC1 after immunization in both wild type and transgenic mice (15). Neither of the adjuvants led to a T cell response or to tumour rejection. Injection of mice with DC pulsed with

the MUC1-derived peptide, however, led to strong T cell immunity and effective tumour rejection in both wild type and MUC1-transgenic mice (15). This study demonstrates both the usefulness of a T cell response against MUC1 and the necessity for delivery of the vaccine to DC to establish a T cell response.

The results of the series of investigations described in this chapter clearly demonstrate that nanospheres containing BLP25 and MPLA-A or MPLA-B can elicit a strong T cell response, while soluble antigen, antigen or adjuvant encapsulated alone or encapsulated adjuvant mixed with soluble peptide could not. It is apparent from the results that only particles containing co-encapsulated antigen and adjuvant can aid DC in overcoming the tolerance of T cells to a self-antigen. No study to date has demonstrated that a single *in vitro* stimulation cycle of T cells with antigen-loaded DC can elicit detectable primary T cell responses against a self-antigen. PLGA nanospheres containing MPLA and BLP25 can break tolerance to a self-antigen and are a strong vaccine candidate for immunotherapy of MUC1$^+$ tumours.

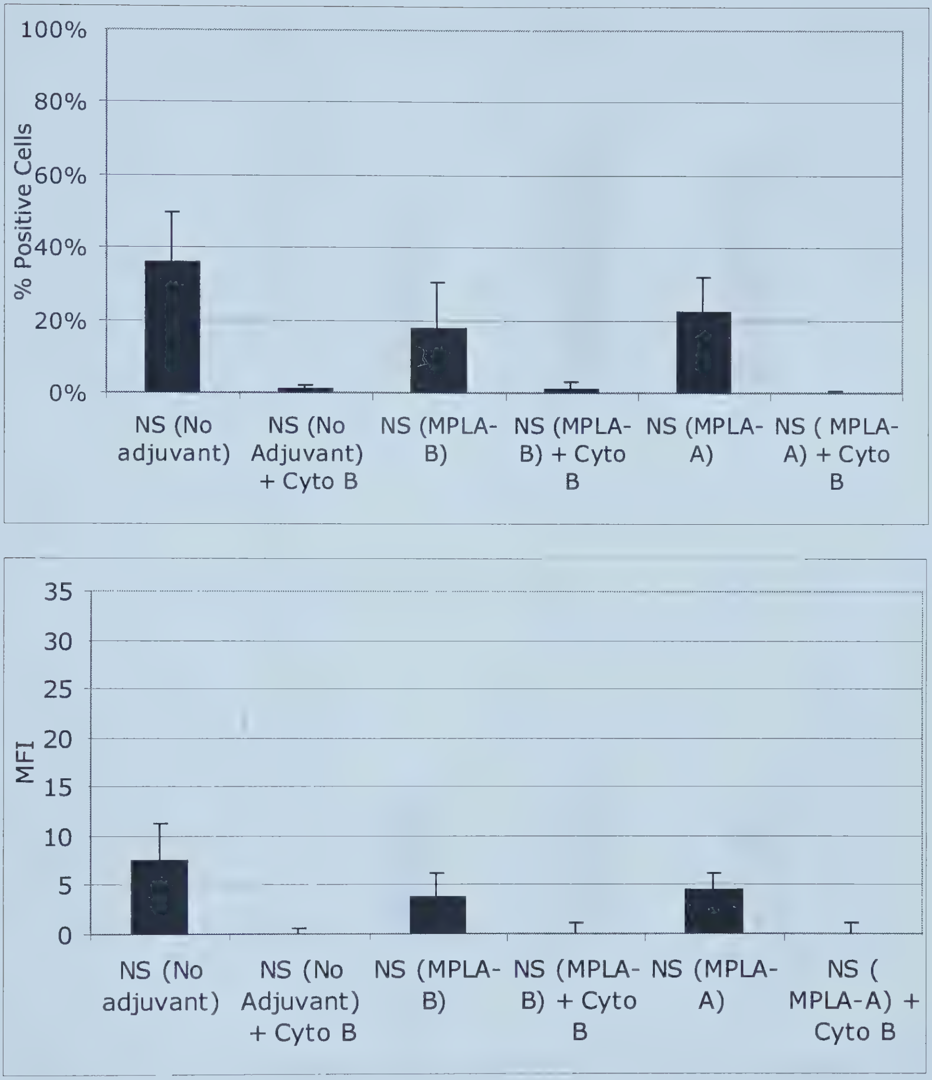


Figure 6-1. Phagocytosis of PLGA Nanospheres. Cells were incubated with nanospheres containing TMR-dextran along with no adjuvant or either MPLA-A or MPLA-B. Control wells were preincubated with a phagocytosis inhibitor, cytochalasin B. Cell-associated fluorescence was measured using a flow cytometer. Results are the average +/- standard deviation for 3 samples from 2 individuals. **A.** The proportion of phagocytic cells in culture as evidenced by the fraction of cells with a fluorescent signal (TMR+). **B.** The average amount of particulate uptake per cell, indicated by the mean fluorescence intensity of the TMR.

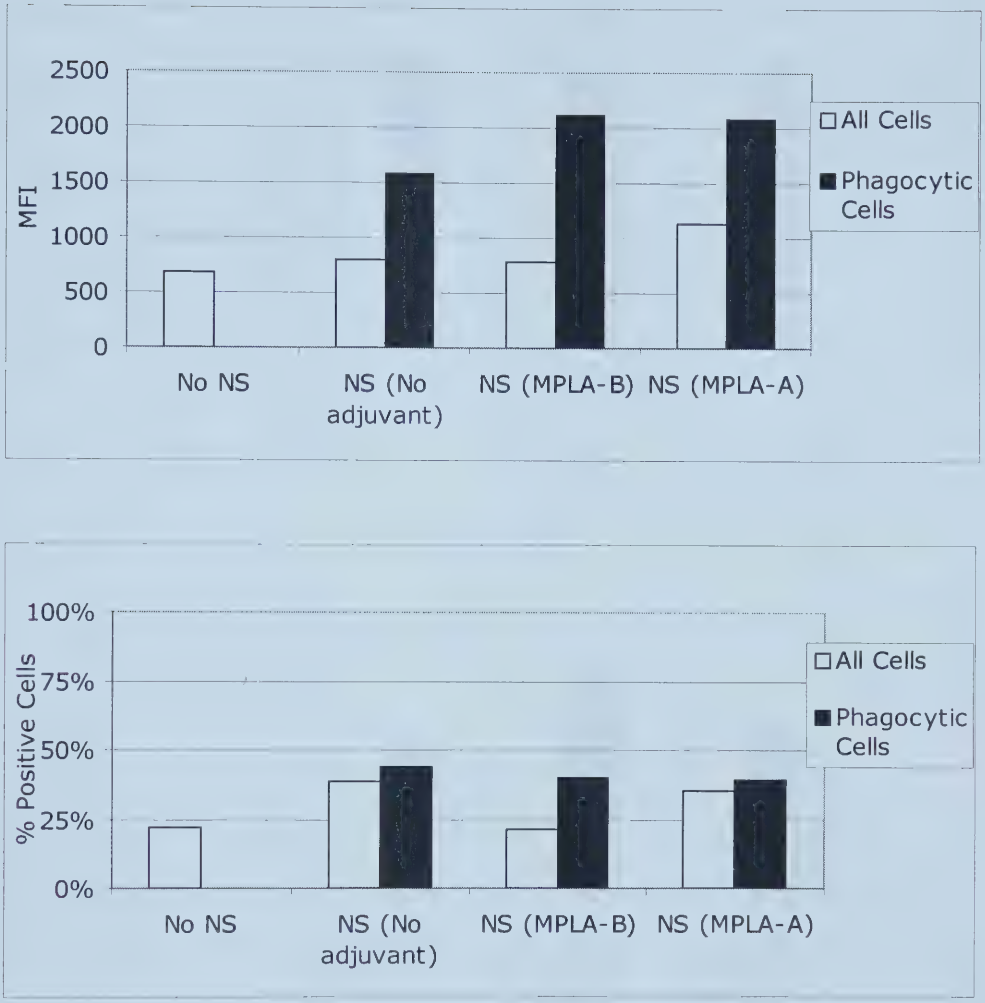


Figure 6-2 (A-B). Cell Surface Marker Expression on DC. After phagocytosis of nanospheres containing TMR-dextran along with either no adjuvant or either MPLA-A or MPLA-B, DC were stained for cell surface markers using FITC labeled Mabs. Expression of markers on all cells (TMR$^+$ and TMR$^-$) was compared to expression on phagocytic cells (TMR$^+$). Results are representative of 1 of 2 individuals. **A.** HLA II Expression. The average level of expression of HLA II molecules as indicated by the mean fluorescence intensity of the FITC signal. **B.** CD80 Expression. The number of cells expressing CD80 indicated by the fraction of cells positive for FITC.

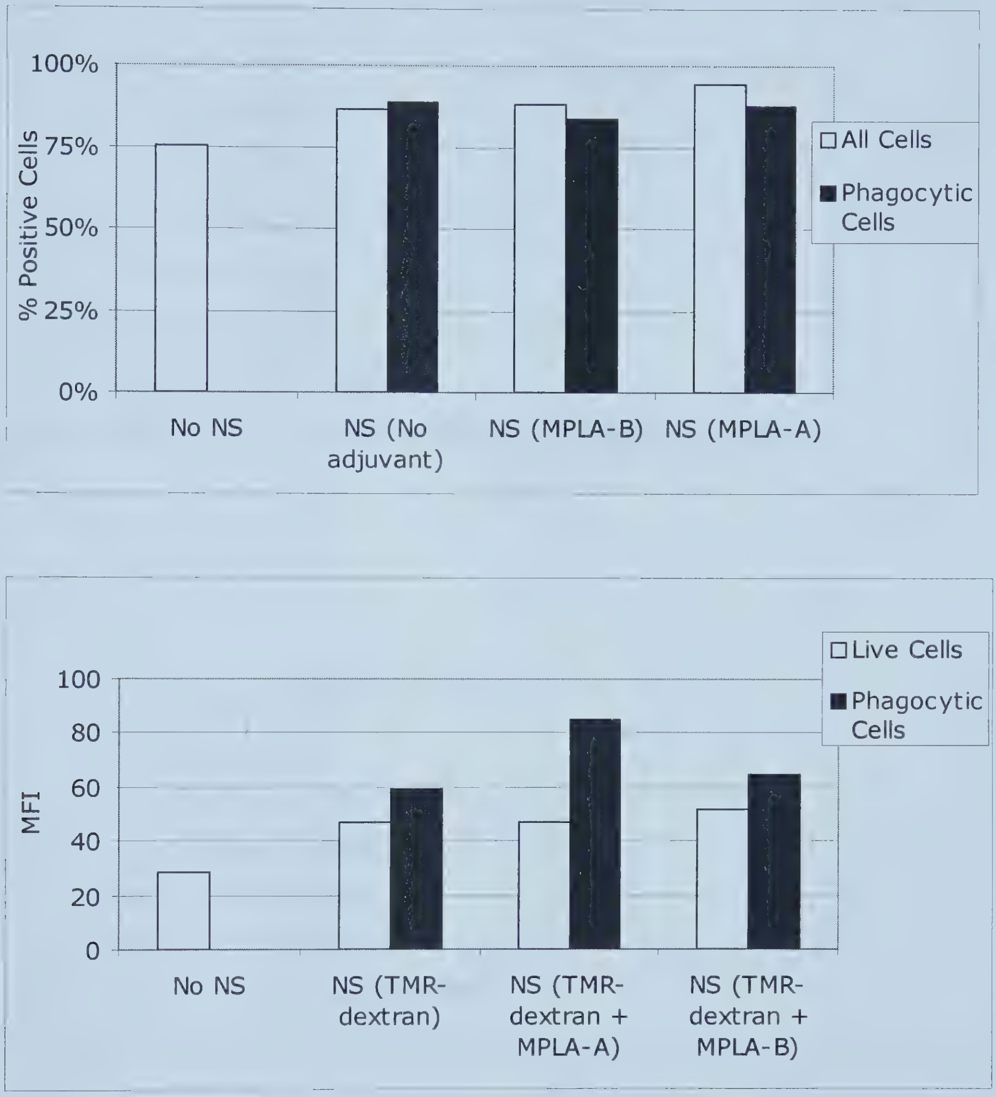


Figure 6-2 (C-D). Cell Surface Marker Expression on DC. After phagocytosis of nanospheres containing TMR-dextran along with either no adjuvant or either MPLA-A or MPLA-B, DC were stained for cell surface markers using FITC labeled Mabs. Expression of markers on all cells (TMR^+ and TMR^-) was compared to expression on phagocytic cells (TMR^+). Results are representative of 1 of 2 individuals. **C.** CD86 Expression. The number of cells expressing CD86 indicated by the fraction of cells positive for FITC. **D.** CD86 Expression. The average level of expression of CD86 molecules as indicated by the mean fluorescence intensity of the FITC signal.

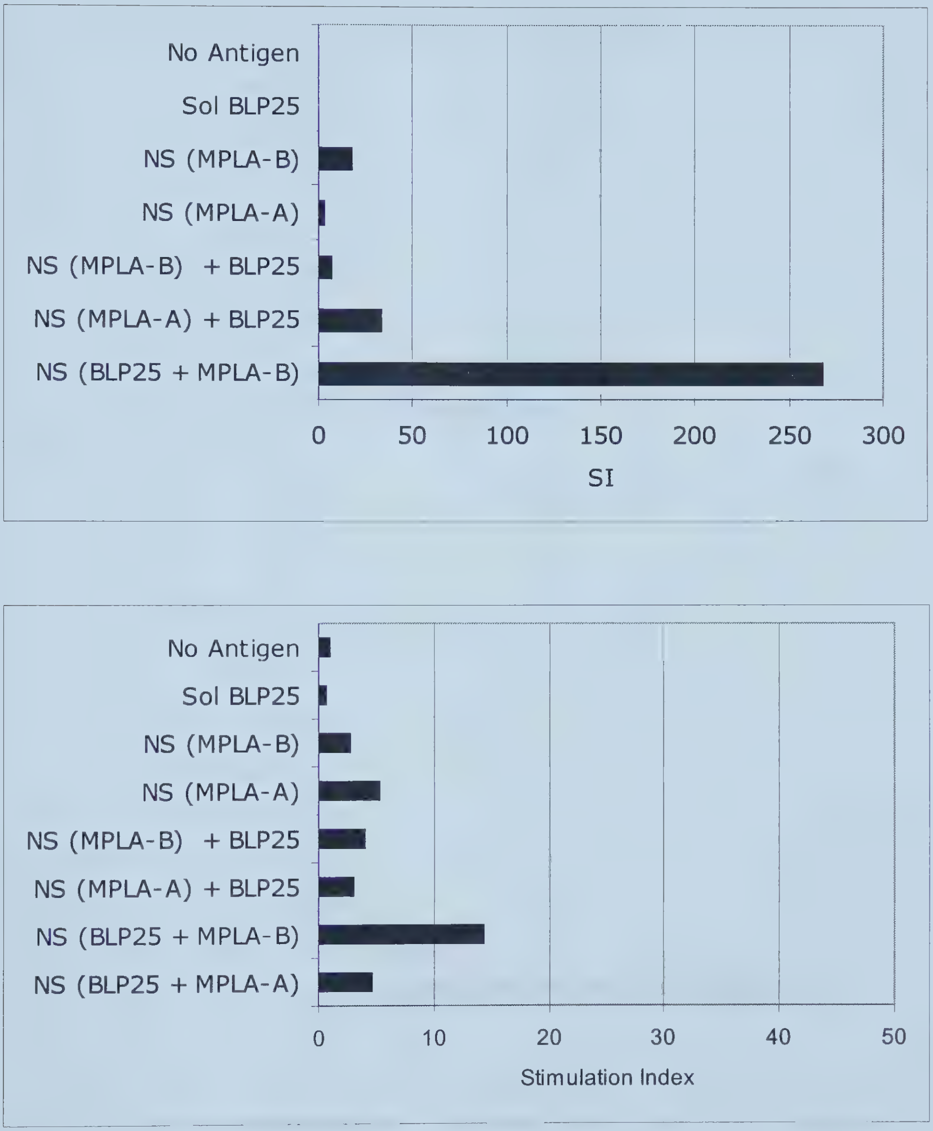
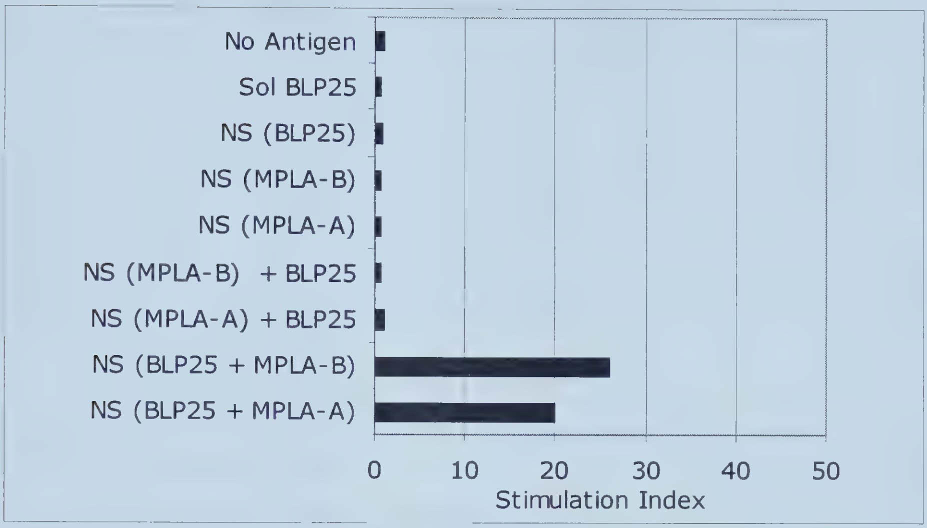


Figure 6-3 (A-B). T Cell Proliferative Response to BLP25. After incubation of DC with different formulations of BLP25, DC were incubated with T cell and the proliferative response was measured by the incorporation of 3H-thymidine. Stimulation Index = average cpm of wells with no antigen/ average cpm of test wells. Results for five separate individuals are shown (A-E).



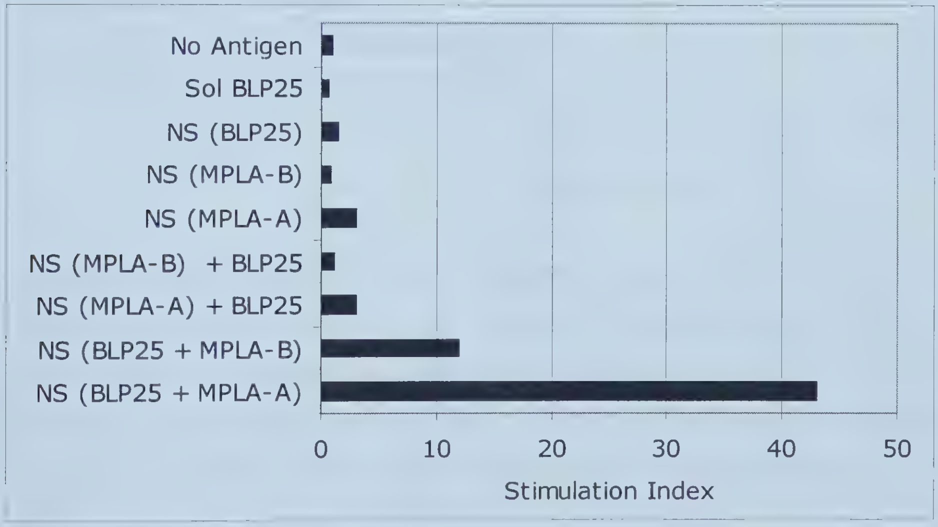


Figure 6-3 (C-D). T Cell Proliferative Response to BLP25. After incubation of DC with different formulations of BLP25, DC were incubated with T cell and the proliferative response was measured by the incorporation of 3H-thymidine. Stimulation Index = average cpm of wells with no antigen/ average cpm of test wells. Results for five separate individuals are shown (A-E).

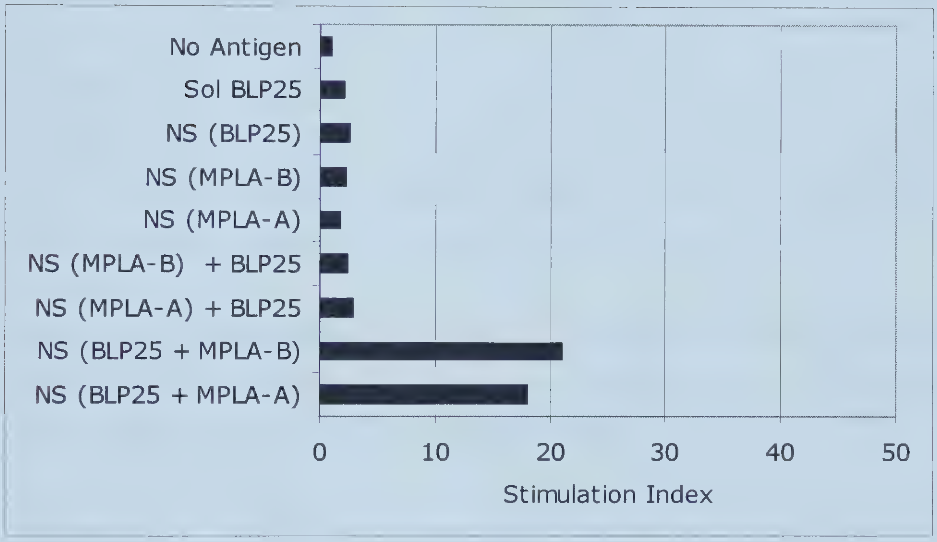


Figure 6-3 (E). T Cell Proliferative Response to BLP25. After incubation of DC with different formulations of BLP25, DC were incubated with T cell and the proliferative response was measured by the incorporation of 3H-thymidine. Stimulation Index = average cpm of wells with no antigen/ average cpm of test wells. Results for five separate individuals are shown (A-E).

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Chapter Seven

Discussion and Conclusions

7.1 Discussion

Vaccination for the treatment of viral diseases and cancer involves the delivery of viral or cancer antigens into the body with the goal of eliciting an antigen-specific immune response that destroys the affected cells while causing minimal or no damage to healthy cells. Delivery of new types of antigens requires, in many cases, the development of novel antigen delivery systems to improve the effectiveness of vaccines. Biodegradable and biocompatible liposomes and polymeric nanospheres have been evaluated for the encapsulation and delivery of a variety of antigens (1-5).

Prior to their use as vaccine delivery systems, liposomal and nanosphere formulations of antigens must be thoroughly characterized. In Chapter 2, liposomal and nanosphere formulations of therapeutically relevant peptides were characterized with respect to size, peptide content, and retention of peptide after 24 hours at 37°C. The size of all formulations was suitable for phagocytic uptake by APC (6-8). Because the dose of antigen can affect the type of immune response elicited by a vaccine formulation (9, 10), it is important to determine the encapsulation efficiency of peptide in the formulation. In Chapter 2, several methods for extraction of peptides from the formulations were examined. For liposomes, the physicochemical properties of the peptide strongly affected the optimal solvent for extraction. In contrast, for nanospheres, peptides with quite different average hydrophobicity values were recovered successfully with the same solvent. The encapsulation efficiencies varied widely in liposomes with the different peptides; in particular, the lipopeptide was the most easily encapsulated. There was no correlation between the average hydrophobicity of the peptide and the encapsulation efficiency; it is likely that the distribution of individual hydrophilic and hydrophobic residues plays more of a role than does the average hydrophobicity of the molecule. In contrast, the limited study done on encapsulation efficiency of peptide into nanospheres demonstrated that method of preparation was more significant than peptide properties in affecting the encapsulation efficiency of the peptide in the formulation.

Finally, the retention of the peptides over 24 hours at 37°C was examined since particulate formulations designed to elicit a cell mediated response are required to retain the antigen within the particles after injection until it is taken up by APC. All formulations retained $\geq$90% of peptide for 24 hours.

The characterization of formulations is a necessary step in the development of new vaccine formulations. The physical properties of the formulations can greatly affect the extent and type of immune response developed against the vaccine *in vivo*. Fully characterizing the formulations can ensure that the size, peptide content, and release characteristics are suitable for the type of immune response desired. In addition, knowing the physical properties of the formulations and studying the immune response generated by the formulation allows further connections between the physical characteristics of the formulations and the immune response elicited, to be established. Following the characterization of the formulations *in vitro*, the immune response to the formulations *in vivo* can be analyzed.

In Chapters 3 and 4, the immune responses to liposomal and nanosphere formulations of $\text{HBcAg}_{129\text{-}140}$ were investigated. It has been shown that patients with chronic hepatitis B and hepatocellular carcinoma have an ongoing antibody response, indicative of a Th2-type response, while patients who resolve the disease develop a cellular immune response with decreased antibody production consistent with a Th1-type response (11). If the immune response in patients could be switched from a Th2-type to Th1-type, the clinical outcome of the HBV infection may be altered. $\text{HBcAg}_{129\text{-}140}$ has been shown to generate a Th2-type response, based on cytokine profile and antibody isotype, in mice of the H-2^b haplotype after immunization with a control formulation of peptide emulsified with complete Freund's adjuvant (CFA) (12). $\text{HBcAg}_{129\text{-}140}$ is a therapeutically relevant antigen that allows investigation into how formulation of the peptide can be used to alter the response to the antigen. Both liposomal and nanosphere formulations of the peptide with MPLA elicited an antigen-specific Th1-type response after

immunization while the CFA formulation did not, however, some questions remain.

The induction of a Th1-type response to $HBcAg_{129-140}$ in naïve mice is a significant step but the situation is not entirely therapeutically. In a chronic hepatitis B patient, there would be an ongoing Th2-type response against the virus that the therapeutic vaccine would have to overcome. In an attempt to mimic this situation, mice were first immunized with a CFA formulation of the peptide in order to prime the mice for a Th2-type response before they were immunized with nanospheres containing peptide and MPLA. Even after the immune system was primed for a Th2-type response, the nanosphere formulation could elicit a strong antigen-specific Th1-type response.

The lack of detection of IL-4 in supernatants of T cell from the CFA immunized mice casts doubt on whether the response in control mice was actually a Th2-type response. There are three possibilities for the lack of IL-4 detection: either there was no IL-4 produced, IL-4 was produced but was exhausted by the cells, or IL-4 was present in the supernatant but the assay used was ineffective. Even if IL-4 was not present in the supernatants, other Th2-type cytokines may be present and need to be analyzed. A lack of IL-4 may also indicate that the CFA formulation elicited a Th0 or a nondefined or mixed response, although the lack of IFN-γ production makes this unlikely. Newer, more sensitive assays for IL-4 and other Th1/Th2 cytokines are available and further investigations should be performed. This peptide has been shown in other laboratories to induce a Th2-type response despite its administration with a strong Th1-inducing adjuvant and therefore can be described as a Th2-inducing peptide. Regardless of the absence of IL-4 in the control supernatants, the ability of the formulations to establish a strong Th1-type response to this peptide is unambiguous.

In this series of experiments, there were no control formulations containing peptide without adjuvant or adjuvant without peptide. Previous work done in

this laboratory demonstrated that both adjuvant and peptide should be incorporated into the same particle for the induction of T cell responses after immunization. *In vitro* work performed using human cells also confirmed this. Thus, MPLA is required for the generation of a Th1-type response to both the liposomal and nanosphere formulations. MPLA is capable of causing the production of IL-12 by DC (13) and the production of IL-12 by DC has been shown to cause a Th1-type response (14). Co-delivery of the antigen and the adjuvant to the same cell enhances the effectiveness of vaccination by allowing the peptide antigen to be presented to T cells by an APC that is producing IL-12 and that is capable of stimulating a Th1-type response. Soluble forms of antigen delivery cannot achieve this co-delivery. *In vitro* investigations involving human DC and T cells can be used to examine the mechanisms of action of the formulations on the induction of immune responses.

Although the evidence supports the theory that a switch in the type of immune response will lead to recovery from hepatitis B, it is yet to be proven. First, the situations in naïve mice and chronically infected humans are significantly different, although the evidence from the mouse studies demonstrating that nanosphere-encapsulated $HBcAg_{129-140}$ can induce a Th1-type response even when the immune system has been primed to respond in a non-Th1 manner is encouraging. It has not yet been demonstrated, however, that the formulation can elicit a response in a patient with chronic hepatitis. Furthermore, it remains to be seen whether a switch in the type of immune response will be sufficient for viral clearance and patient recovery.

In addition to the implications for the immunotherapy of chronic disease, these investigations have a more general relevance. Viral diseases and malignancies are believed to require a Th1-type response in order for a cure to be affected. The success of liposomal and nanosphere formulations in inducing a Th1-type response against a hepatitis B peptide may be applied to other antigens.

DC are the key APC responsible for the stimulation of T lymphocytes (15) and for this reason therapeutic vaccine formulations must be designed to deliver antigen to DC *in vivo*. Particulate delivery systems mimic microorganisms, allowing for natural targeting of the vaccine to APC and optimal uptake by APC leading to endosomal and cytoplasmic delivery of antigen for processing and presentation on both class I and II MHC molecules. Since the uptake of PLGA nanospheres by DC will prove crucial for the success of the vaccine formulation, investigations into the ability of DC to phagocytose nanospheres and the effects of this phagocytosis on DC need to be investigated. In Chapter 5, the ability of human DC to phagocytose PLGA nanospheres was established.

The ability of DC to phagocytose and process antigen is highly dependent upon the stage of DC differentiation (16, 17). Immature cells residing in the tissue are highly phagocytic and are less efficient at stimulating T lymphocytes. Following maturation, DC cease phagocytosis and evolve into efficient APCs that express high levels of costimulatory molecules (16, 17). The results in Chapter 5 and 6 demonstrate that at day 6, 7 or 8 in culture, the vast majority of cells were able to uptake particles while on day 3, only about 40% of cells phagocytosed nanospheres. At this early stage in culture, many of the monocyte precursor cells have not fully differentiated into DC, accounting for the lower proportion of phagocytic cells in the population. The high proportion of phagocytic cells at Day 6, 7, and 8 indicates that the cells in these cultures are not fully mature since they have not ceased phagocytosis.

Costimulatory molecule expression is upregulated as DC mature (18-21). After phagocytosis of a vaccine formulation *in vivo*, it is necessary for the DC to become activated and traffic to the lymph node where they can present antigen to the T cells. For this reason, the effect of PLGA nanosphere formulations on the maturation state of the cell is an essential area of

investigation. It is believed that MPLA can supply an activation signal to DC that causes them to mature (22) and it is possible that nanospheres themselves will cause maturation of DC. At day 8 in culture, no changes in cell surface marker expression could be discerned between phagocytic and nonphagocytic cells or between cells receiving MPLA-A, MPLA-B, or no adjuvant. However, at day 3, both MHC II molecules and CD86 were upregulated on the surface of phagocytic cells. It is likely that at day 8, the effect of culture conditions on the maturation state of the cells masked any formulation effects. This should be taken into account in future investigations.

The fluorochrome that was encapsulated in nanospheres for use in the phagocytosis studies was not optimal for flow cytometry applications. Because of this, the levels of fluorescence were quite low. At the time these studies were performed, TMR-dextran was the best of a short list of possible compounds that could endure the harsh formulation process without significant loss of fluorescence activity. As new compounds become available, phagocytosis studies could be performed that do not underestimate the amount of cell-associated fluorescence observed.

A thorough understanding of the delivery of PLGA nanospheres to DC will allow for the refining of vaccine formulations to optimize uptake. In addition, the effect of formulations on the DC can be determined. Knowledge of the effect of phagocytosis of nanospheres on DC allows researchers to tailor formulations to stimulate DC into the most effective T cell-stimulators possible. Furthermore, investigations into the uptake of formulations by DC and the effect of these formulations on the cells can be used to elucidate potential mechanisms for the success of the vaccines *in vivo*.

As well as the characterization of uptake and the effect of formulations on the expression of cell surface molecules by DC, the effect of formulations on the ability of DC to stimulate a T cell response needs to be established. The

preliminary investigations in Chapter 6 demonstrate that, after uptake of antigen encapsulated with MPLA in nanospheres, DC can activate a strong T cell response to a self-antigen. This response requires that adjuvant and antigen be contained in the same particle, confirming *in vivo* results.

The ability of DC to process and present a tumour-associated antigen from PLGA nanospheres and stimulate a T cell response is significant but it was performed *in vitro* and results cannot always be extrapolated to *in vivo* conditions. However, these *in vitro* investigations are essential for several reasons. First, there is the necessity of performing *in vitro* work in order to examine mechanisms behind the ability of formulations to elicit a response. In addition, it is crucial for cancer immunotherapy that investigations occur using human cells and preliminary investigations must be performed *in vitro*.

Because cancer antigens are self-antigens, T cells are tolerized to them and breaking this self-tolerance is the first requirement for immunotherapy of malignancies. Although self-tolerance was not specifically examined in these studies, it is likely, based on published reports, and the lack of a MUC1-specific recall response *in vitro*, that the experimental subjects are tolerant to MUC1. In this model, PLGA nanospheres broke tolerance to a self-antigen and stimulated a strong T cell response to the antigen *in vitro*. It is possible that the factors regulating tolerance against antigens are considerably different in the *in vivo* situation and further investigations are needed. The ability of PLGA nanospheres to elicit an *in vitro* T cell response against a self-antigen after a single stimulation remains a highly significant result that emphasizes the potential value of this delivery system for antigen delivery.

The development of therapeutic vaccines requires the identification of an antigen and the appropriate formulation of antigen to elicit the optimal response, followed by investigation of the abilities and limitations of the formulation. Particularly in the case of peptide antigens, large numbers of potential antigens may need to be screened for activity. The selection of the

most appropriate formulation may also require significant investigation. A judicious combination of *in vivo* animal studies and *in vitro* human cell studies can supply a great deal of information about the effectiveness of vaccine formulations and the mechanisms behind the successes and failures. This information can then be used to refine the therapeutic vaccines. In this dissertation, a combination of animal and human cell studies were used to evaluate liposomal and nanosphere formulations of therapeutically relevant peptides and to begin ascertaining the mechanisms of the activation of the immune response.

7.2 Conclusions

1) Liposome and nanosphere formulations of a Th2-inducing peptide can elicit an antigen-specific Th1-type response to the peptide.
2) In a situation somewhat closer to the therapeutic situation, even after the immune system has been primed for a Th2-type response, immunization with $HBcAg_{129-140}$ and MPLA in nanospheres can elicit an antigen-specific Th1-type response.
3) DC, the key APC, phagocytose PLGA nanospheres.
4) In early immature DC, uptake of PLGA nanospheres causes an upregulation of MHC II molecules and the costimulatory molecule, CD86, indicating progression to an activated state.
5) After phagocytosing PLGA nanospheres, DC can process and present the encapsulated peptide to T cells and stimulate a strong T cell response. Delivery of peptide derived from a self-antigen to DC in nanospheres can lead to the breaking of tolerance to a self-antigen.

7.3 Future Avenues of Investigation

Since all work with the hepatitis B antigen was performed in mice, investigations need to be performed in a human system. It is not yet known if the immune system of an individual with chronic hepatitis B can elicit a response against the vaccine. Investigations using peripheral blood cells from patients with chronic hepatitis B should be performed in order to

determine whether or not an antigen-specific T cell response can be elicited from T cells *in vitro*. In addition, the $HBcAg_{129-140}$ formulations need to be tested in a disease model. Although the evidence supports the theory that a switch in the type of immune response will lead to disease eradication, it is not yet apparent whether the recovery from disease actually occurs after immunotherapy.

Because most work on uptake of delivery systems has been performed using $M\phi$, systematic studies on the effect of formulation parameters on the uptake of nanospheres by DC should be performed. Size, lactic acid: glycolic acid ratio, and polymer MW should all be examined for their effect on uptake. In addition, investigations into the effect of different formulations on the DC should be performed. Further delineation of how to optimize both uptake and DC activation by altering formulation parameters will enable the refining of vaccine formulations.

The work done on the activation of human T cells by DC *in vitro* in this dissertation was preliminary and further studies are needed. First, HLA-typing of donors should be performed and the ability of the nanosphere formulation of BLP25 to elicit a response in genetically diverse individuals should be studied. Second, the type of T cell response needs to be determined. Blocking antibodies against class I and class II MHC would allow the identification of the type of T cell responding. In addition, cytokine analysis of the culture supernatants could be used to further identify the type of T cell response. Third, the effect of altering formulation parameters on the extent and type of response should be performed and the mechanisms involved could be elucidated. Finally, it is necessary to determine if effective CTL are generated in response to the PLGA nanosphere formulation. This can be determined by investigation whether or not the activated T cells can specifically kill MUC1 expressing tumour cells.

There are further steps that can be taken to develop immunotherapies against malignancies and chronic viral infections. Information about the physicochemical properties of microorganisms that are effective at elicing immune responses *in vivo* can be exploited to design better delivery systems. In addition, as a more thorough understanding of the intricacies of the immune system is constructed, it will become clear what requirements must be met by a vaccine in order to establish an effective immune response. The targeting of antigen delivery systems to DC, activation of DC by the delivery systems, and the effective presentation of antigen to T cells can all be enhanced through careful delivery system development.

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